The Topological Distribution of Olfactory Receptor Neuron Axons in the Olfactory Bulb Glomeruli of the Rat: A Confocal Microscopic Study with DiI Staining

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Finally to Delores Montoya, your big smile and warm greeting were just what I needed when everything around me seemed to be going poorly.

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ABSTRACT

THE TOPOLOGICAL DISTRIBUTION OF OLFACTORY RECEPTOR NEURON AXONS IN THE OLFACTORY BULB GLOMERULI OF THE RAT: A CONFOCAL MICROSCOPIC STUDY WITH DII STAINING.

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The olfactory system encompasses three anatomical sites. Initial detection begins in the posterior aspect of the nasal cavity where specialized olfactory receptor neurons transduce odorants into neural signals. These signals are then transmitted to the central nervous system where they undergo elaborate processing within spherical shaped regions of neuropil in the first relay station of the olfactory pathway, the olfactory bulb glomeruli. The resulting bulbar signal is then propagated to numerous cortical structures. Although the precise code employed by the olfactory system to discriminate between individual odorants remains to be elucidated, the existence of an odortopic map is supported by converging lines of evidence. The singularly unique pattern of activity across the glomerular layer of the olfactory bulb produced by a particular odorant appears to encode its identity. The activity of any single glomerulus thus represents the fundamental unit of this code. Furthering an understanding of olfactory processing, therefore, requires a detailed analysis of the anatomy of those processes contributing to the complex neuropil of the olfactory bulb glomerulus, especially that of the primary afferent projections from the neuroepithelium. It has been previously demonstrated in Golgi-impregnated specimen that single olfactory

receptor neuron axons occupy only a limited region of the glomerular neuropil (Hálasz and Greer, 1993). In the present study, we extend these findings by performing a confocal examination of the distribution of DiI stained axons and fascicles as they approach, penetrate, and arborize within glomeruli. Olfactory bulbs from two week old Sprague-Dawley rats were immersion fixed in 4% paraformaldehyde, implanted with 2-3 DiI crystals in the olfactory nerve layer, incubated at room temperature and in the dark for an average of 3.5 weeks, and cut transversely at 100μm with a vibratome. Thin optical images, 0.5-1μm, were then captured in series with a BioRad 600 confocal laser and reconstructed in two and three dimensions for analysis. Single axons were easily resolved and exhibited both en passant varicosities and terminal boutons among intraglomerular axonal collaterals, which ranged from 2-6. Consistent with Golgi-impregnated samples, individual axons appeared to arborize within only a small proportion of the glomerulus. Most significantly, fascicles of primary afferent axons were demonstrated to distribute within spatially restricted domains, rather than ramifying homogeneously throughout the glomerulus. The terminal arbors of olfactory receptor neuron axons from the same fascicle appear to colocalize within a limited region of the glomerular neuropil, mostly segregated from the terminal arbors of those axons from a different fascicle. In addition, the terminal arbors of primary afferent fibers appear to be concentrated in the peripheral shell of the glomerulus with few penetrating into the glomerular core. These data thus support a sub-compartmental organization of the olfactory bulb glomerulus. Glomerular sub-compartments may represent the segregation of parallel afferent pathways from the neuroepithelium to the olfactory bulb, or alternatively, could be involved in the individualized processing of differential odorant information within a single glomerulus.

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INTRODUCTION

The sensory systems of any organism exert a powerful influence on behavior and thereby contribute to survival. Although human beings tend to focus on the aesthetic quality of the senses, the evolution of the five senses was driven by the primitive need of any living thing to be acutely aware of its environment. The olfactory system, one of the two chemical senses, is devoted to the perception of volatile molecules that make their way into the nasal cavity from the surrounding environment. Its pivotal role in mate selection, in appetitive behaviors, in identifying territorial boundaries, and in recognizing the approach of predators has been demonstrated in non-Homo sapiens. Although humans seem to be unaware of these primitive drives of olfaction, odorants and the neural responses they direct must obviously influence human behavior as well. Only recently, however, have investigators begun to subject the olfactory system to the same detailed study afforded other sensory systems. The anatomy, cytology, physiology, biochemistry, and neural networking involved in odorant detection has begun to be elucidated. The complex code used by the olfactory system to discriminate odors, however, has proved more difficult to decipher. Unlike the precise topography employed by the visual and somatosensory systems to map sensory space, the olfactory system seems to lack this same type of point-to-point processing. Yet it must possess a precise mechanism by which it can discriminate between the thousands of distinct stimuli called odorants. Elucidating this code is essential to further understand the olfactory system and its complex processing of sensory information.

Olfactory System I: General Overview and Anatomy

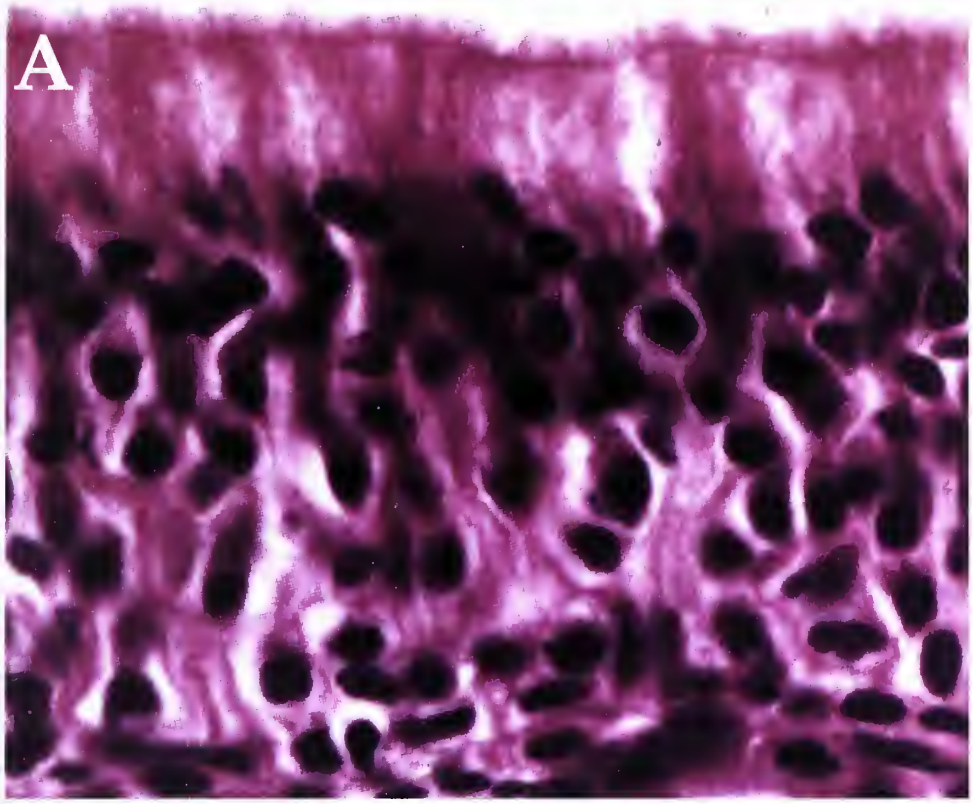
The olfactory system encompasses three anatomical sites, each uniquely designed to contribute to the processing of odorant information. Initial detection of odorants begins in the posterior aspect of the nasal cavity, where specialized sensory cells transduce the physical phenomena of odorants into neural signals. These signals are then transmitted to the central nervous system where they undergo elaborate processing within the first relay station of the olfactory pathway, the olfactory bulb. The resulting bulbar signal is then propagated to numerous cortical structures where the subjective sensation of odor is perceived and from which an appropriate response can be directed. Although the precise code employed by the olfactory system to discriminate between individual odorants is still being elucidated, much of the anatomy and physiology of the olfactory system, in general, and its three component parts, specifically, have been described in detail.

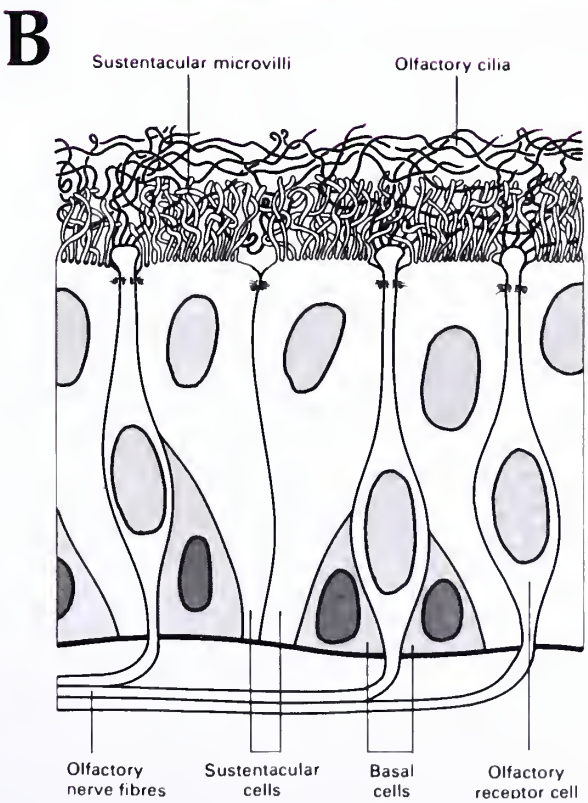
Olfactory Neuroepithelium: histology, cytology, and odorant transduction

The mucosal lining of the nasal cavity, although uniform in its pseudostratified epithelial architecture, exhibits a diversity of cellular constituents divided between two functionally distinct zones: an anterior respiratory epithelium and a posterior olfactory neuroepithelium. Composed of ciliated cells, goblet cells, brush cells, and basal cells, the anterior respiratory epithelium warms, humidifies, and filters inspired air (Ross and Romrell, 1989). Functionally, it is part of the respiratory system. The posterior olfactory neuroepithelium contains supporting sustentactular cells, basal cells, and highly specialized olfactory receptor neurons (Figure 1). It is

Figure 1:

Olfactory Neuroepithelium: Hematoxylin and Eosin Micrograph (A) and Schematic Diagram (B). Composed of olfactory receptor neurons, sustentacular cells, and basal cells, the mucosa covering the posterior aspect of the nasal cavity is functionally a component of the nervous system. It is the site of the initial events in odorant detection and processing. Ascending from the apical pole of each olfactory receptor neuron is an apical dendrite which, upon reaching the mucosal surface of the epithelium, expands into a dendritic knob and gives rise to several long cilia. Converging lines of evidence suggest that these cilia are studded with odorant receptor molecules which, upon binding complementary ligands, induce a second messenger cascade and the generation of an action potential in the olfactory receptor neuron. Extending from the basal pole of each olfactory receptor neuron, an unmyelinated axon passes into the cranial cavity to distribute within the olfactory bulb, thus forming the olfactory or first cranial nerve. Turning-over approximately every 30-120 days, olfactory receptor neurons are continually replaced by daughter cells derived from the basal cells, a neuroblast population. Sustentacular cells, although previously thought to serve only a supportive role, are now recognized to perform a host of functions in the neuroepithelium. See text for further details. (Adapted from Wheater et al., 1987)


A


B
Sustentacular microvilli
Olfactory cilia
Olfactory nerve fibres
Sustentacular cells
Basal cells
Olfactory receptor cell

functionally a component of the nervous system, for it is these olfactory receptor neurons which perform the initial events in odorant detection and discrimination.

The olfactory receptor neurons are identified by their approximately 5μm diameter ovoid soma, apical dendrite extending to the mucosal surface, and basal axon which passes through the ethmoid bone to distribute within the olfactory bulb. Although derived from the olfactory placode and hence true neurons, olfactory receptor neurons exhibit certain unique characteristics. They are indeed the only neurons with direct exposure to the external environment. Perhaps as a consequence, they are not a static population but undergo continual turnover every 30-120 days depending on species and environmental conditions (Graziadei and Monti-Graziadei, 1979; Costanzo and Becker, 1986). Replacement cells have been demonstrated to arise from the mitotic activity of the basal cells. Following transection of the olfactory nerve, the basal cells shows an increase in both the incorporation of tritiated thymidine as well as the number of mitotic figures (Costanzo and Graziadei, 1987). Found singly or in clusters along the basement membrane of the neuroepithelium, the basal cells are approximately 6μm diameter cells and represent a neuroblast population, whose daughter cells differentiate into mature olfactory receptor neurons. The position of an olfactory receptor neuron somata within the olfactory neuroepithelium is thought to be a reflection of its age: the soma of young cells lie in proximity to their basal cells of origin and the basement membrane, while the soma of older cells are found at more superficial layers.

Regardless of its position, the somata of each olfactory receptor neuron sends a single, 1-2μm diameter, unbranching apical dendrite to the mucosal surface. At its tip, the dendrite expands into a terminal dendritic knob or

olfactory vesicle which gives rise to numerous long, immobile cilia that blanket the epithelial surface (Moran et al., 1982; Farbman and Menco, 1986; Morrison and Costanzo, 1990). These cilia are identical in their microtubular cytoskeleton to those of any other cell, but their plasma membrane is endowed with a host of intramembranous particles unique among cilia. Recognizing that the cilia are the only portion of the olfactory receptor neuron exposed to the external environment, investigators postulated that these intramembranous particles represent the site of odorant receptor proteins (Menco and Farbman, 1985a, 1985b).

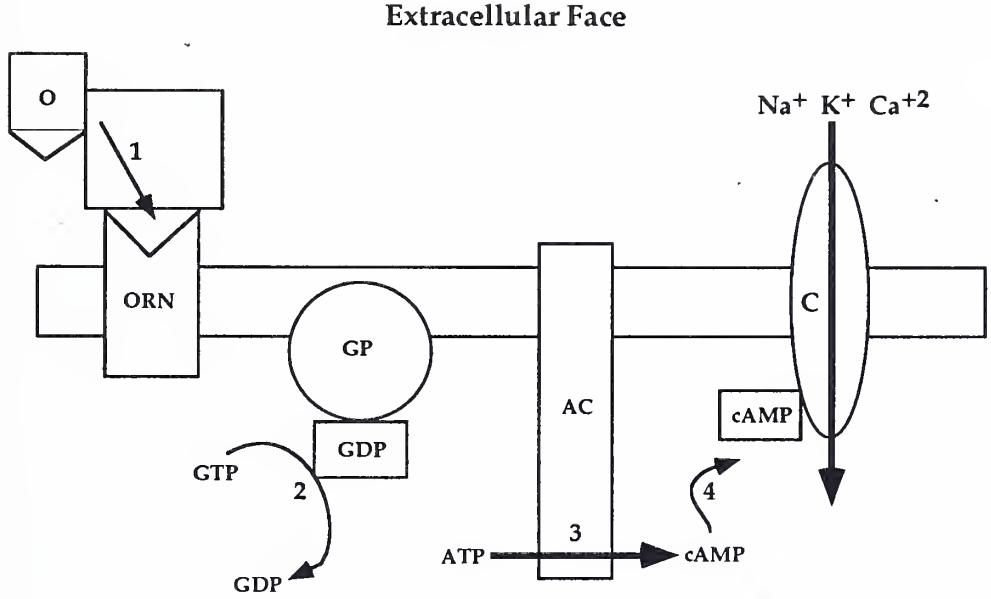
Although neither biochemical nor functional studies have yielded any direct confirmation of this theory, the existence of a receptor mediated G-protein-dependent mechanism for odorant transduction, signal generation, and detection is supported by converging lines of evidence. In fish, the binding of known olfactory stimuli to isolated cilia, supporting the existence of specialized receptor molecules on the cilia's surface, has been observed (Rhein and Cagan, 1980, 1983). The removal of the cilia from the olfactory receptor neurons of frogs is associated with the elimination of all odorant-induced responses (Bronshtein and Minor, 1977). Clearly the initial events in odorant detection involve the cilia of olfactory receptor neurons. Additionally, biochemical studies, which were initially successful in demonstrating the odorant induced activation of a second messenger cascade in olfactory receptor neurons (Pace et al., 1985; Sklar et al., 1986; Boekhoff et al., 1990; Breer et al., 1990), have more recently identified olfactory specific guanyl-nucleotide-binding proteins (G-protein), G_{olf} and G_o, and a type III adenylate cyclase which would be responsible for the production of second messenger molecules in the cilia (Jones and Reed, 1989; Pfeuffer et al., 1989; Jones, 1990). These second messengers could serve to open specific cationic

channels, thereby depolarizing the cell and eliciting an action potential. In fact, an olfactory specific cyclic nucleotide-gated channel has been localized to the plasma membrane of the cilia of olfactory receptor neurons (Nakamura and Gold, 1987; Dhallan et al., 1990). When bound to a cyclic nucleotide, its permeability to sodium, potassium, and calcium (Reed, 1992) creates a net conductance which depolarizes the cell to threshold and produces a propagating action potential. Taken together, these observations are consistent with a model of odorant transduction whereby the odorant induced generation of second messengers, via odorant association with G-protein linked receptors on the cilial surface, serves to activate specific cationic channels and thereby generate an action potential in the olfactory receptor neuron (Figure 2). The existence of these odorant receptors, however, remains to be authoritatively established.

Guided by the transduction model outlined above, investigators have succeeded in identifying a family of genes whose transcripts possess the stipulated characteristics of an odorant receptor protein and may, therefore, represent the odorant receptors expressed on the cilia of olfactory receptor neurons. Using polymerase chain reaction (PCR) and gene cloning, they were able to demonstrate a multigene family, belonging to the superfamily of seven transmembrane domain G-protein linked receptors, which is expressed only in the olfactory neuroepithelium (Buck and Axel, 1991), and perhaps only in olfactory receptor neurons (Ressler et al., 1993). As members of the G-protein linked superfamily, these receptor proteins clearly share the sequence homology which would allow them to associate with G_{olf} or G_o and thereby trigger the odorant induced second messenger cascade observed in other studies (Pace et al., 1985; Sklar et al., 1986; Boekhoff et al., 1990; Breer et al., 1990). The presence of sequence diversity in the presumed odorant binding

Figure 2:

Schematic Diagram of Odorant Transduction Pathway. Converging lines of evidence have implicated a guanyl-nucleotide-binding protein (G-protein), second messenger cascade for odorant transduction in the olfactory receptor neurons of the neuroepithelium. Investigators have identified olfactory specific G-proteins (GP), adenylate cyclase (AC), and cyclic nucleotide-gated channels (C) in olfactory receptor neurons. In addition, there appears to be a multigene family, belonging to the superfamily of G-protein linked receptors, whose expression is limited exclusively to olfactory receptor neurons. Although there has been no direct evidence of odorant binding or G-protein activation by the transcripts of this multigene family, the four-step transduction pathway outlined here is generally accepted. (1) The association of odorant (O) with its complementary receptor (ORN) induces a conformational shift in the receptor protein thereby inducing the activation of a G-protein (GP). (2) The exchange of GTP for GDP in the guanyl-nucleotide binding site of the G-protein triggers dissociation of its active sub-unit which then activates adenylate cyclase (AC). (3) Adenylate cyclase catalyzes the conversion of ATP to cAMP. (4) cAMP binds to the cyclic nucleotide binding site of a cyclic nucleotide-gated ion channel (C) which opens and conducts potassium, calcium, and sodium into the cell. This influx of positive current serves to depolarize the olfactory receptor neuron to threshold and trigger an action potential. See text for further details. [O=odorant; ORN=olfactory receptor neuron; GP=G-protein; AC=adenylate cylcase; C=cyclic nucleotide-gated channel]


Extracellular Face
O
1
ORN
GP
GDP
GTP
2
GDP
AC
3
ATP
cAMP
4
cAMP
C
Na+ K+ Ca+2
Intracellular Face

site, transmembrane helices 3, 4, and 5, would restrict the association of each receptor protein, from the enormous repertoire of possible odorants, to a limited ensemble of specific, complementary molecules.

> The possibility that each member of this large family of seven transmembrane proteins is capable of interacting with only one or a small number of odorants provides a plausible mechanism to accommodate the diversity of odor perception (Buck and Axel, 1991).

Sequence analysis of these variable regions reveals sub-families of odorant receptors, each with greater homology among their own members than between the members of different sub-families (Buck and Axel, 1991). The considerable sequence homology within one sub-family suggests a somewhat uniform association with one particular class of odorants. Individual receptors of any sub-family, therefore, may function to refine odorant recognition by responding best to subtle differences between related stimuli (Buck and Axel, 1991; Ressler et al., 1993). Despite the inability to demonstrate ligand binding or second messenger activation by the protein transcripts of any member of this multigene family (Reed, 1992), it is highly probable that they form the odorant receptors expressed on olfactory receptor neuron cilia. Their association with specific, complimentary odorants would serve to activate the proposed odorant transduction pathway and lead to the production of a signal in the olfactory receptor neuron which is propagated to the central nervous system along its axon.

The axon of the olfactory receptor neuron is a 0.1-0.4μm in diameter, unmyelinated axon which extends from the basal pole of the soma. Together, these axons form the olfactory nerve, the first cranial nerve. After fasciculating into mesaxons of approximately 200 individual processes and penetrating the epithelial basement membrane, they travel into the lamina

propria and become ensheathed by the encircling membrane of a special class of Schwann cells. Although the axons of the peripheral nervous system are consistently insulated by Schwann cells, those Schwann cells participating in the olfactory nerve possess certain unique characteristics. They express the astrocytic protein glial fibrillary acidic protein (GFAP) but no laminin, fibronectin, or vimentin, and therefore chemically resemble astrocytes (Barber and Lindsay, 1982; Schwob and Gottlieb, 1986). The manner in which they encase a tightly packed mesaxon, rather than individual axons, is atypical for a peripheral nerve but may allow for ephaptic interactions between neighboring axons (Getchell and Shepherd, 1975b; Bliss and Rosenberg, 1979; Gesteland, 1986; Eng and Kocsis, 1987). The ensheathed mesaxons coalesce into even larger fascicles and pass through the many foramina of the cribiform plate of the ethmoid bone to distribute within the most superficial layer of the olfactory bulb, the olfactory nerve layer.

An additional cell population observed in the neuroepithelium includes the supporting or sustentacular cells. They are easily distinguished from the olfactory receptor neurons in three ways: 1) each cell extends from the basement membrane to the mucosal surface, 2) their apical surface is covered with microvilli, and 3) their nuclei are distributed in a linear array in the epithelium rather than diffusely through all layers. Having neither an axon nor a dendrite and deriving their embryological origin from the non-nervous ectoderm of the olfactory placode, these cells are thought to serve mainly a supportive function. Histological sections clearly demonstrate the close approximation of ascending apical dendrites of olfactory receptor neurons and surrounding, rectangular-shaped, sustentacular cells as well as the presence of junctional complexes. Yet these cells are not so limited in

their function. One author summarizes the numerous suggestions of other investigators as follows:

> Their functions may include: (a) insulation of olfactory receptor cells, (b) a glialike function in the neuroepithelium, (c) secretion (of acidic, sulfated, and/or neutral mucopolysaccharides from the apical pole of the cell surface), (d) transepithelial transport of molecules, and (e) guides for developing neurons and neural processes (Morrison and Costanzo, 1992).

Sustentacular cells may even play a role in odorant processing. After disassociating from their receptors on olfactory receptor neurons, odorants may be bound-up by sustentacular cells thus preventing the re-excitation of odorant-induced pathways (Shepherd, 1989).

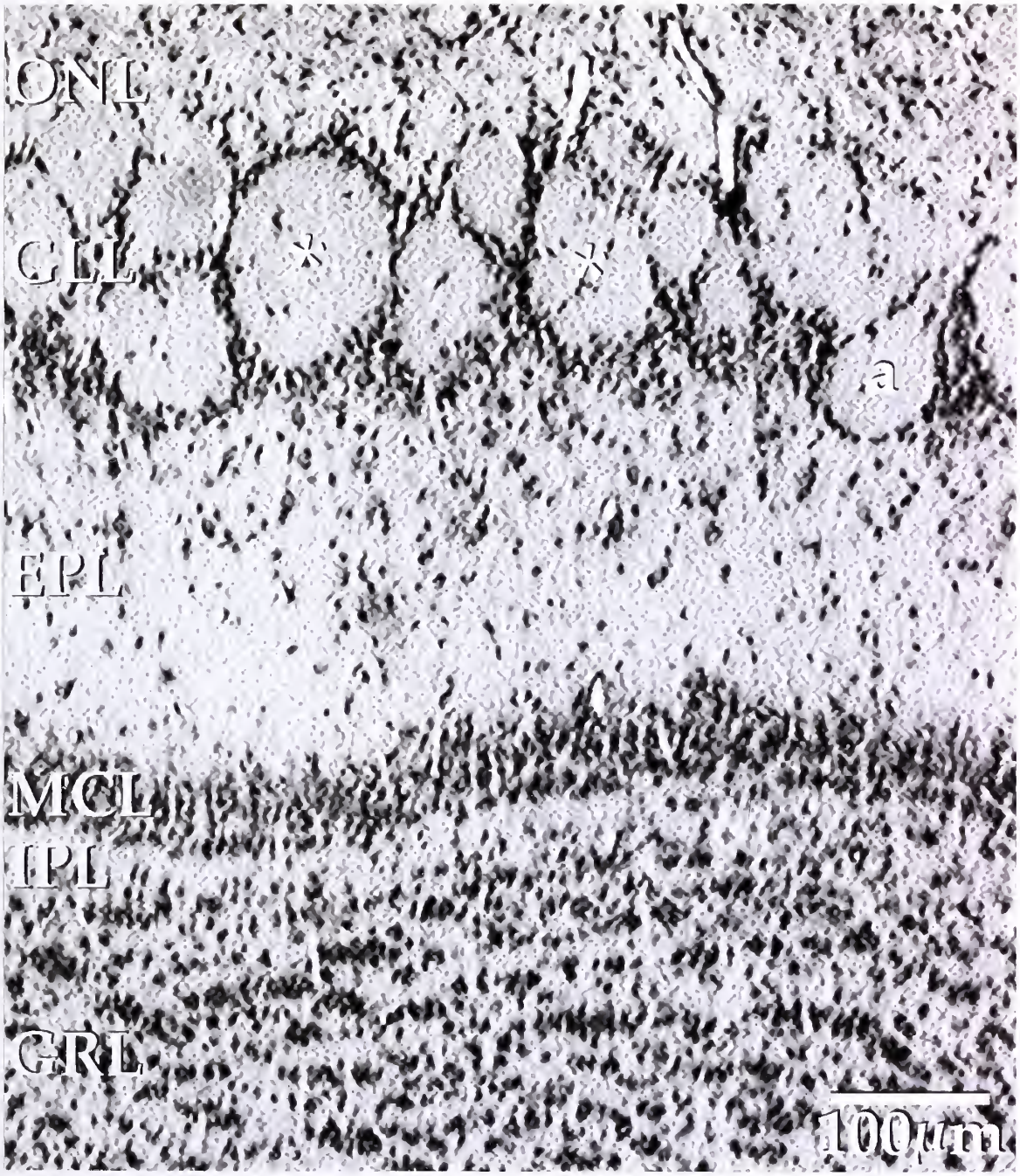
Olfactory Bulb: histology and cytology

The olfactory bulb, an outgrowth of the forebrain, is a highly organized, laminated structure where the axons of the olfactory receptor neurons make the first synapse in the processing of olfactory information. Its symmetric layers are easily identified in cresyl-violet stained sections (Figure 3). From superficial to deep, they are: olfactory nerve layer, glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer, and granule cell layer .

After passing through the neuroforamina of the cribiform plate of the ethmoid bone, the primary afferent fibers of olfactory receptor neurons distribute widely over the surface of the olfactory bulb, forming the olfactory nerve layer. These axons course caudally along the bulb and dive, as discrete fascicles, into the next deeper layer of the olfactory bulb to terminate within specific regions of neuropil called glomeruli. Although they travel the entire

Figure 3:

Cresyl-violet Stain of the Olfactory Bulb. In this figure, the laminar organization of the olfactory bulb is evident. After passing through the many neuroforamina of the cribiform plate of the ethmoid bone, the axons of olfactory receptor neurons distribute within the most superficial layer of the olfactory bulb, the olfactory nerve layer (ONL), and then dive into the next deeper layer, the glomerular layer (GRL), to terminate within individual areas of neuropil called glomeruli (asterisks and a). Each glomerulus is surrounded on three-sides by the soma of periglomerular cells, an intrabulbar interneuron population, as observed in the glomerulus marked: a. Within the external plexiform layer (EPL) and mitral cell layer (MCL) are the soma and secondary dendrites of the principal or projection neurons of the olfactory bulb, the tufted and mitral cells. These cells transmit a long apical dendrite through the external plexiform layer and into individual glomeruli where they synapse with the axons of olfactory receptor neurons and the dendrites of periglomerular cells (see text). Overlapping these secondary dendrites in the external plexiform layer are the apical dendrites of granule cells whose cell bodies make-up the most internal lamina of the olfactory bulb, the granule cell layer (GRL). Complex synaptic interactions have been identified between the apical dendrites of granule cells and the secondary dendrites of mitral and tufted cells (see text). [ONL=olfactory nerve layer; GLL=glomerular layer; EPL=external plexiform layer; MCL=mitral cell layer; IPL=internal plexiform layer; GRL=granule cell layer; asterisks=glomeruli; a=glomerulus in which the calyx-like organization of periglomerular cells is demonstrated]


ONL
GLL
a
EPL
MCL
IPL
GRL
100μm

distance from somata to glomerulus without branching (Allison and Warwick, 1949; Pinching and Powell, 1971a, 1971b), once within the confines of one of these spherical structures, approximately 100μm in diameter in small mammals, they arborize extensively. Axonal varicosities and terminal boutons, the sites of synaptic transmission, are observed in abundance among these arbors.

Periglomerular or juxtaglomerular cells are an intrabulbar interneuron population found exclusively in the glomerular layer of the olfactory bulb. Forming calyx-like structures, their 6-8μm diameter soma surround and demarcate individual areas of glomerular neuropil (a in Figure 3). In addition, their dendritic processes penetrate and ramify within the adjacent glomerulus. The axons of periglomerular cells travel laterally in the extraglomerular neuropil of the glomerular layer, as far as five glomeruli away, but do not leave the olfactory bulb. Thus periglomerular neurons are true short axon cells. The activity carried by their axons to distant targets in other glomeruli may play a role in intrabulbar interglomerular circuits (Pinching and Powell, 1971a, 1971b).

The primary projection or principal neurons of the olfactory bulb are the mitral and tufted cells. In many species, including mammals, the mitral cells are more easily identified owing to the linear arrangement of their 15-30μm diameter soma in the mitral cell layer, just deep to the external plexiform layer. Extending without branching from each somata is an apical dendrite, 2-12μm in diameter, that passes through the external plexiform layer and into a single glomerulus where it then arborizes extensively. Each mitral cell also possesses several laterally directed secondary dendrites, 1-8μm in diameter and 500-800μm in length, which distribute, ramify, and terminate within the external plexiform layer and whose sub-laminar location serves to

distinguish specific sub-sets of mitral cells (Macrides and Schneider, 1982; Mori et al., 1983; Orona et al., 1984) (Figure 4A). Type I mitral cells (M1 in Figure 4A) give rise to secondary dendrites which radiate through the external plexiform layer at its deepest level, adjacent to the mitral cell layer. The secondary dendrites of type 2 mitral cells (M2 in Figure 4A) ascend to a more superficial strata of the external plexiform layer and distribute in proximity to the glomerular layer.

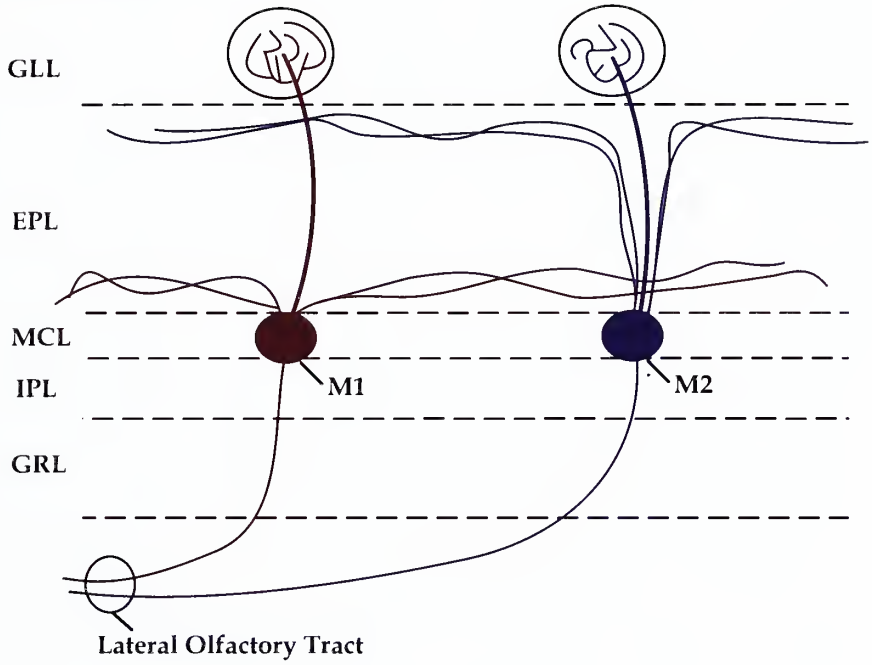
Unlike the laminar organization of mitral cell soma, the cell bodies of tufted cells are dispersed throughout the external plexiform layer. Sub-populations of tufted cells are distinguished by anatomical and cytological criteria (Figure 4B). While internal tufted cells are those 15-30μm soma located in the deepest strata of the external plexiform layer in proximity to the mitral cell layer, external tufted cells (T_e in Figure 4B) are found in the most superficial portion of the external plexiform layer just deep to the glomerular layer. The main population of tufted cells, the middle tufted cells (T_m in Figure 4B), are those with soma located at intermediate levels of the external plexiform layer and with a diameter of 15-20μm. They possess several secondary dendrites which disseminate laterally within the superficial external plexiform layer. Like mitral cells, all tufted cells possess an apical dendrite which ascends through the external plexiform layer to arborize within an individual glomerulus.

The myelinated axons of mitral and tufted cells transmit the efferent signals from the olfactory bulb. Together they form the lateral olfactory tract at the posterolateral surface of the olfactory bulb, with one exception. The axons of external tufted cells project exclusively to intrabulbar sites and, therefore, may participate in local association circuits (Schoenfeld et al., 1985). In addition, intrabulbar collaterals from those axons destined for the lateral

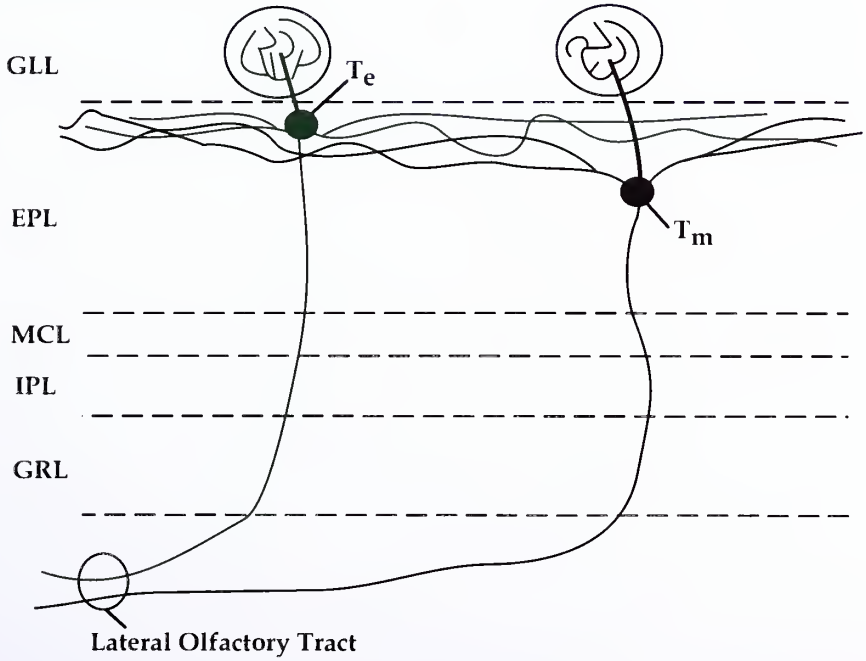
Figure 4:

Diagram of the Cyto-architecture of Sub-populations of Mitral (A) and Tufted Cells (B). (A) The sub-populations of mitral cells are distinguished by the sub-laminar distribution of their secondary dendrites in the external plexiform layer (EPL). While the secondary dendrites of Type I mitral cells (M1) are distributed in the most internal portion of the external plexiform layer adjacent to the mitral cell layer (MCL), type II mitral cells send their secondary dendrites to the most superficial region of the external plexiform layer to distribute just deep the to the glomerular layer (GLL). **(B)** The sub-populations of tufted cells are distinguished by the sub-laminar position of their soma in the external plexiform layer. While the soma of external tufted cells (T_e) are located just deep to the glomerular layer, the main population of tufted cells, the middle tufted cells (T_m), occupy a more intermediate position in the external plexiform layer and distribute secondary dendrites in the superficial external plexiform layer. Not shown are the internal tufted cells which lie just external to the mitral cell layer. [GLL=glomerular layer; EPL=external plexiform layer; MCL=mitral cell layer; IPL=internal plexiform layer; GRL=granule cell layer]

A: Mitral Cells



B: Tufted Cells



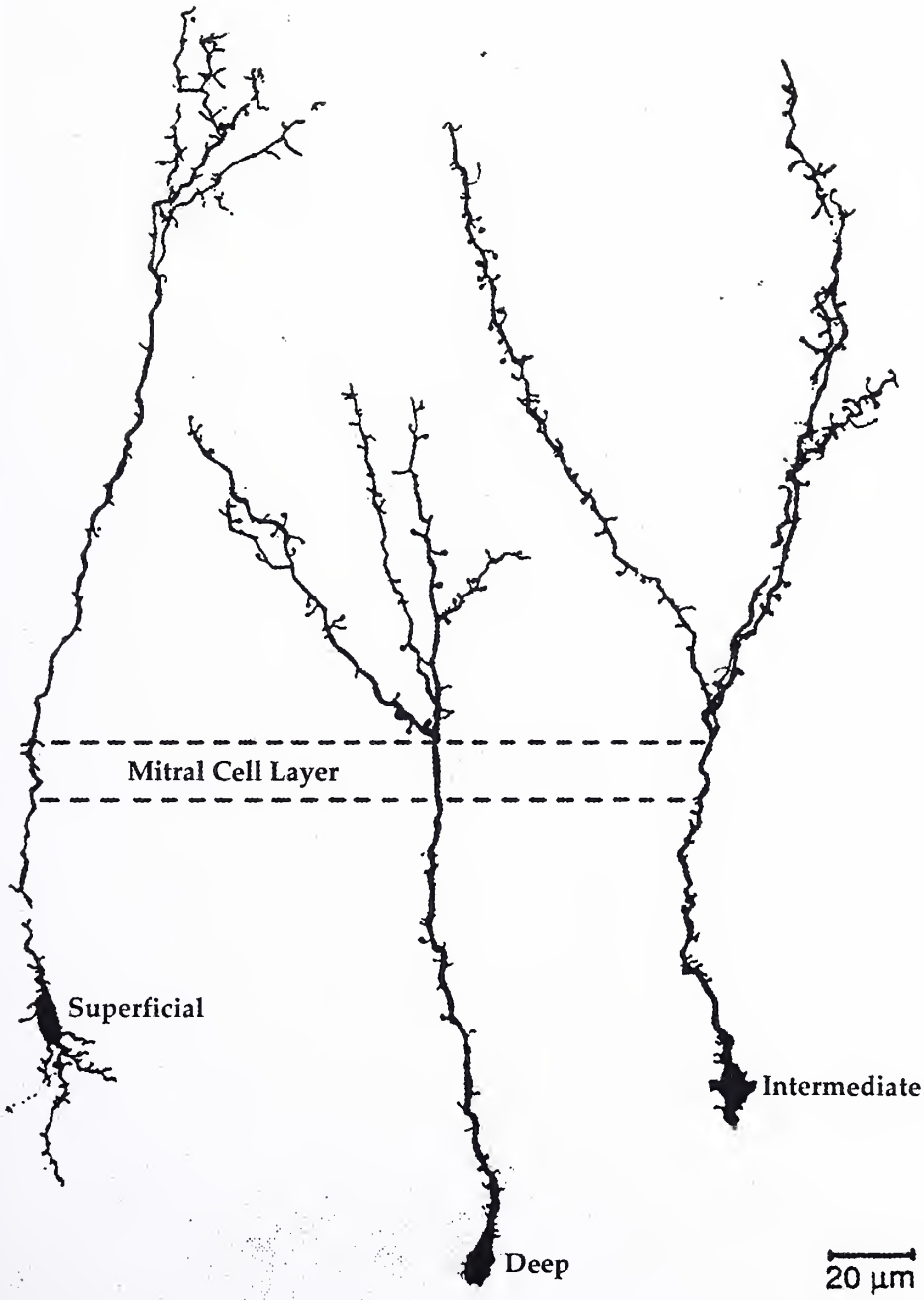
olfactory tract may play a similar role. Within the internal plexiform and granule cell layers, mitral and tufted cell axons can be observed to branch and provide intrabulbar collaterals which distribute widely among the soma of granule cells (Kishi et al., 1984; Orona et al., 1984).

Like the periglomerular cells, the granule cells are a population of intrabulbar interneurons. Their 6-8μm soma are found in clusters throughout the deepest layer of the olfactory bulb, the granule cell layer. Although anaxonic, granule cells possess both an apical dendritic process that ascends into and distributes within the external plexiform layer and a smaller basal dendritic process which ramifies intrinsically in the granule cell layer. Both are studded with numerous spines or gemmules, the site of synaptic interactions. Sub-populations of granule cells have been identified and are characterized by the level at which their apical dendrite arborizes and distributes within the external plexiform layer (Mori et al., 1983; Orona et al., 1983; Macrides et al., 1985; Greer, 1987) (Figure 5). Although the apical dendritic process of intermediate granule cells can be found throughout the external plexiform layer, superficial and deep granule cells send their apical dendrite exclusively to the most superficial and deep strata of the external plexiform layer, respectively.

This sublaminar organization of the apical dendrites of subpopulations of granule cells parallels that of the secondary dendrites of subpopulations of mitral and tufted cells. In the superficial external plexiform layer, the dendritic processes of type 2 mitral cells, middle tufted cells, and superficial granule cells overlap in their distribution. In the deep external plexiform layer, there is colocalization of the dendritic processes of type 1 mitral cells and deep granule cells. Lacking any specific sublaminar organization, intermediate granule cells overlap all other fibers in the external plexiform

Figure 5:

Cyto-architecture of Sub-populations of Granule Cells. The granule cells are an intrabulbar interneuron population whose soma can be found in clusters in the deepest lamina of the olfactory bulb, the granule cell layer. Anaxonic, granule cells possess apical and basal dendritic processes. Sub-populations of granule cells are differentiated by the sub-laminar distribution of their apical dendrites within the external plexiform layer. While the apical dendrites of intermediate granule cells can be identified throughout the external plexiform layer, superficial and deep granule cells send their apical dendrites into superficial and deep strata of the external plexiform layer, respectively. (Adapted from Shepherd and Greer, 1990)


Mitral Cell Layer
Superficial
Intermediate
Deep
20 µm

layer. Since synaptic interactions are possible only between overlapping processes, there appears to be an apparent targeting of specific sub-populations of bulbar cells for each other. Some investigators have proposed that this segregation of external plexiform fibers represents a level of parallel processing inherent to olfactory bulb circuits (Shepherd and Greer, 1990). As in other sensory systems, it is conceivable that each sub-population of principal neurons is involved in a unique set of interactions with specific bulbar elements and conveys some independent quality of the stimulus to the cortex.

The identification of numerous corticobulbar pathways between wide areas of the brain and the olfactory bulb suggest a degree of central nervous system control over bulbar circuits (Macrides and Davis, 1983; Mori, 1987; Scott and Harrison, 1987). Both the ipsilateral and contralateral anterior olfactory nuclei send afferent fibers diffusely throughout the olfactory bulb. Axons of pyramidal neurons in piriform cortex branch to form collaterals which terminate in the granule cell layer. Many brainstem centers, including the horizontal limb of the diagonal band, the locus coeruleus, and the raphe nucleus, provide input to the glomerular and granule cell layers. Within the glomerular layer, the cholinergic fibers from the diagonal band terminate on periglomerular targets, while the noradrenergic fibers from the locus coeruleus and the serotoninergic fibers from the raphe nucleus synapse exclusively within the glomerular neuropil. The precise targets of these diverse afferent fibers and the role they play in olfactory processing remains to be elucidated. Similarly, the function and synaptic interactions of olfactory bulbar short axons cells, a diverse population of intrabulbar neurons dispersed throughout the external plexiform and granule cell layers, continues to be investigated.

Cortical Substrates of Olfaction: targets of the lateral olfactory tract

The lateral olfactory tract contains most of the axons of the principal neurons of the olfactory bulb, the mitral and tufted cells. Although these two cell populations probably represent parallel pathways in odorant processing, their combined output targets the anterior olfactory nucleus, the olfactory tubercle, the amygdala, and rostral piriform cortex. The only sites of segregated input from these two sets of efferents is entorhinal cortex and caudal piriform cortex, which exclusively receive the output of mitral cells (Scott, 1981; Prince, 1987). Considering that piriform cortex is designated primary olfactory cortex, it is not surprising that it receives the combined input from both mitral and tufted cells in a topographical manner. In the cortex, the axons of mitral and tufted cells synapse with the apical dendrites of pryamidal cells, each of which transmits a long axon to numerous cortical and sub-cortical sites. Hence, wide areas of cortex seem to be involved in odorant detection, discrimination, and in directing an appropriate response.

Olfactory system II: Odorant Processing and Discrimination

After detecting the presence of an odorant, the single most important task of the olfactory system is to identify its unique qualities and to encode them in a system by which the particular odorant can be discriminated from the host of possible stimuli. This is indeed the process performed by all sensory systems: detection, discrimination, and finally subjective perception. Yet unlike the visual, auditory, and somatosensory systems which must

encode a spatial dimension as well, the olfactory system is only concerned with the nature of the offending stimulus.

> Relieved of the necessity to encode information about the spatial localization of the sensory stimulus, the olfactory system of mammals may use the spatial segregation of sensory input solely to encode the identity of the stimulus itself (Buck and Axel, 1991).

In fact, a great deal of evidence now indicates that olfactory receptor neurons can be divided into functionally distinct classes, each of which transmits their combined input to a limited area of the olfactory bulb, activating perhaps a few specific glomeruli. It is the differential activity across the glomerular layer of the olfactory bulb that is postulated to be the code employed by the olfactory system for odorant discrimination. The complex synaptic architecture of the olfactory bulb is then uniquely responsible for regulating the afferent signals from the neuroepithelium and refining the efferent signals to cortex, thereby establishing this code.

Synaptic Architecture of the Olfactory Bulb: an input-output processor

Within the glomerular neuropil of the olfactory bulb, olfactory receptor neuron axons form synaptic interactions with the dendrites of mitral, tufted, and periglomerular cells. Motivated by its significance as the site of the first synapse in the olfactory pathway, intense investigation has succeeded in both elucidating the complex synaptic architecture of the olfactory bulb and in furthering an understanding of odorant processing. It is now recognized that the olfactory bulb represents a complex input-output processor (Shepherd and Greer, 1990). Afferent information from the neuroepithelium and efferent projections to the cortex are modified and regulated by highly specialized local

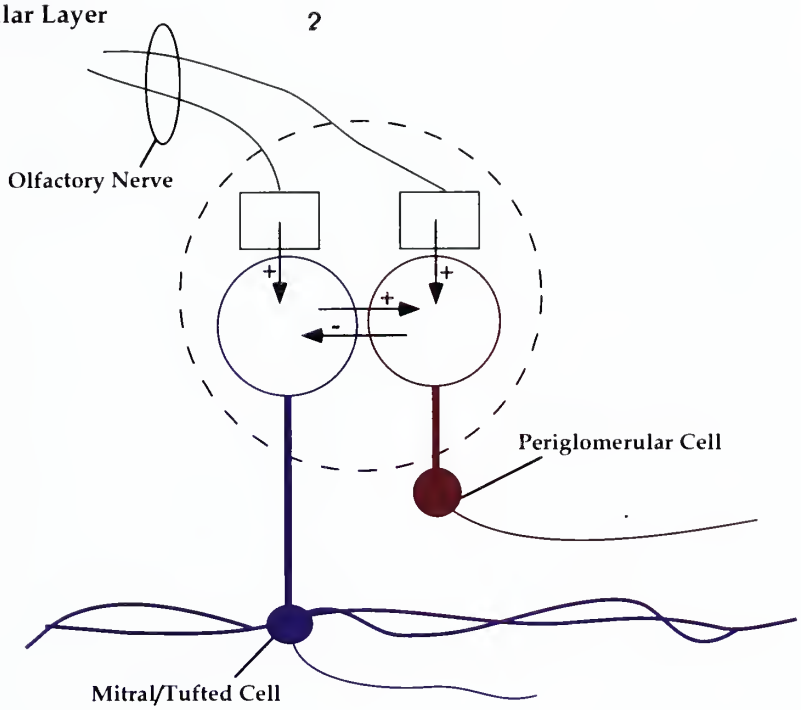
circuit interactions between the principal neurons of the olfactory bulb, mitral and tufted cells, and intrabulbar interneurons, periglomerular and granule cells. The synaptic organization of the olfactory bulb anatomically segregates these functions to two discrete lamina: input processing at the level of the glomerulus and output processing in the external plexiform layer (Shepherd and Greer, 1990).

The major contributors to the neuropil of a single glomerulus include the overlapping arbors of approximately 25,000 olfactory receptor neuron axons, the apical dendrites of approximately 25 mitral and 75 tufted cells, and the dendritic arbors of an undetermined number of periglomerular cells (Shepherd, 1972, 1977). Within these structures, olfactory receptor neuron axons make asymmetric, excitatory axodendritic synapses with the dendrites of mitral, tufted, and periglomerular cells (Pinching and Powell, 1971a, 1971b; White, 1973) (Figure 6A). The degree to which individual afferent fibers discriminate between and target specific post-synaptic populations, versus the degree to which they distribute homogeneously, remains to be elucidated. Yet irrespective of any targeting which may occur, "the synaptic action of the olfactory receptor cell axon onto any one postsynaptic dendrite will also affect the remaining dendrites" (Greer, 1991). This is a direct result of the fundamental synaptic organization of the glomerulus, the synaptic triad. The synapses formed by olfactory receptor neuron axons not only transmit primary olfactory information to the apical dendrites of the principal neurons of the olfactory bulb, they also excite periglomerular cells, thereby activating highly specialized reciprocal dendrodendritic interactions between periglomerular dendrites and the apical dendrites of mitral and tufted cells (Figure 6A). EM serial reconstructions of the ultrastructural features of these interactions are consistent with an excitatory synapse between the principal

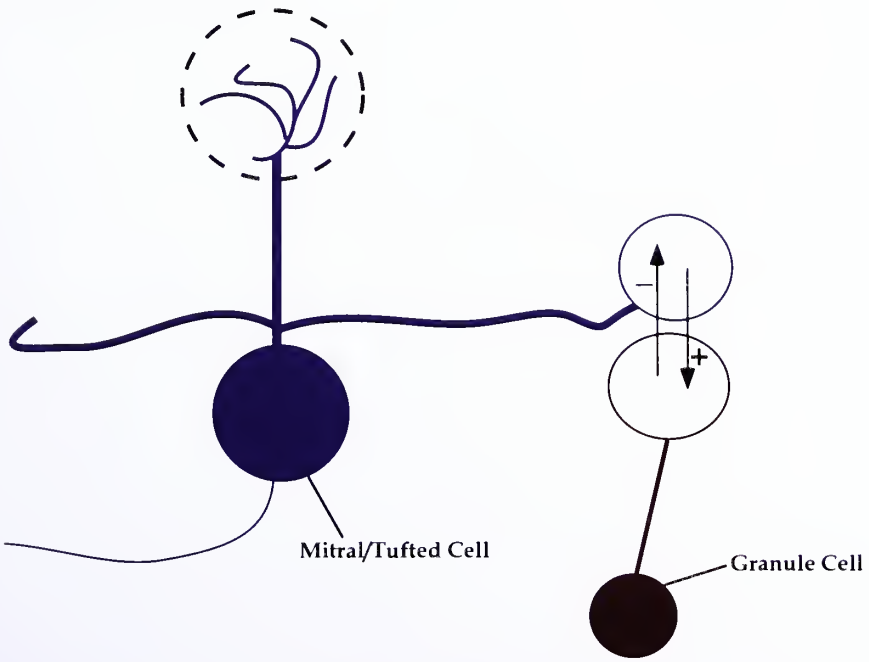
Figure 6:

Schematic Diagram of the Synaptic Architecture of the Olfactory Bulb: Synaptic interactions in the glomerulus (A) and the external plexiform layer (B). **(A)** Afferent information from the olfactory neuroepithelium is transmitted to the olfactory bulb via the axons of olfactory receptor neurons. Within individual glomeruli, these primary afferent fibers form excitatory, type II synapses with the dendrites of mitral, tufted, and periglomerular cells. This not only transmits olfactory information to the principal or projection neurons of the olfactory bulb, it also serves to activate the modulatory effect of periglomerular cells. The periglomerular cell dendrite is involved in reciprocal dendrodendritic interactions with the apical dendrites of mitral and tufted cells. Ultrastructural examination reveals an inhibitory synapse between the periglomerular cell and mitral or tufted cell and an excitatory synapse in the reverse direction. The periglomerular cell also transmits a long axon through the glomerular layer where it influences the activity in other glomeruli (not shown). Overall, the synaptic interactions of the glomerular layer appear to be involved in regulating the degree of afferent-induced activity in the olfactory bulb. **(B)** In the external plexiform layer, the secondary dendrites of mitral and tufted cells form reciprocal dendrodendritic interactions with the apical dendrites of granule cells. These synapses have been characterized as excitatory from mitral or tufted cell to granule cell and inhibitory in the reciprocal direction. Since granule cells are anaxonic, their sole output is this inhibitory effect on the activity of the principal neurons of the olfactory bulb which is otherwise conveyed to cortical structures. [+=excitatory synapse; -=inhibitory synapse]

A: Glomerular Layer



B: External Plexiform Layer



neuron and the periglomerular cell and an inhibitory synapse in the reciprocal direction (Pinching and Powell, 1971a, 1971b; White, 1972, 1973; Getchell and Shepherd, 1975a). The action of these dendritic interactions may be to modify the primary afferent induced activity of mitral and tufted cells, and thus modulate the excitatory input to the olfactory bulb.

The excitation of periglomerular cells by primary afferent fibers also activates an important intrabulbar interglomerular circuit. The axons of periglomerular cells travel great distances within the non-glomerular neuropil to make excitatory (Shepherd, 1963; Freeman, 1974) and inhibitory (Getchell and Shepherd, 1975a, 1975b) synapses with the somata and dendrites of other periglomerular cells and the apical dendrites of principal neurons. Hence, the afferent input to a single glomerulus can have wide ranging effects across the glomerular layer. Extrapolating from other sensory systems, investigators have postulated a role in lateral inhibition between glomeruli for this interglomerular circuit (Pinching and Powell, 1971a, 1971b). If primary olfactory afferents project to a diverse set of glomeruli, the efferents to cortical structures could be limited to the output of a sub-set of these glomeruli via the inhibitory effects of periglomerular cells.

At the level of the external plexiform layer, the secondary dendrites of mitral and tufted cells and the apical dendrites of granule cells overlap in a sub-population specific manner. Here they form reciprocal dendrodendritic interactions similar to those observed in the glomerulus (Hirata, 1964; Rall et al., 1966) (Figure 6B). In this mutual exchange, the mitral or tufted cell synapse with a granule cell is excitatory, while its reciprocal, granule cell-to-mitral or tufted cell is inhibitory (Prince and Powell, 1970). Since granule cells are anaxonic, this inhibitory effect on the activity of mitral and tufted cells is their sole output and, like the activity of periglomerular cells, may participate

in lateral inhibition. By modulating olfactory bulb efferents, the granule cell may play an important role in shaping principal neuronal output to cortical targets.

Although previously considered merely a relay station between the neuroepithelium and cortex, it is now apparent that the olfactory bulb may play an important role in the processing of olfactory stimuli. Its synaptic architecture is clearly not designed for the mere transfer of odorant signals, but instead to perform an essential step in odorant processing. Several lines of evidence suggest that the olfactory bulb glomerulus may represent the fundamental unit in the discriminatory code employed by the olfactory system. The highly specialized synaptic interactions intrinsic to the olfactory bulb, both in the glomerulus and external plexiform layer, would obviously contribute to establishing and refining this code.

Discriminatory Code of the Olfactory System

Each olfactory receptor neuron expresses about one of the approximately 100 members of the putative odorant receptor gene family (Ngai et al., 1993; Ressler et al., 1993). Activity in any one odorant receptor-specific olfactory receptor neuron population, therefore, results from the binding of its particular complementary stimulus. This, some have stated, simplifies the discriminatory task of the olfactory system; distinguishing between individual odorants involves recognizing which population of olfactory receptor neurons have been activated (Vasser et al., 1994). However studies with experimental odorants fail to demonstrate a singularly specific interaction of an odorant with a particular receptor, and instead indicate that one odorant can interact with a host of receptors. Odorants are recognized to

have multiple functional epitopes, each able to bind to a limited number of receptors, with varying affinity. Each odorant receptor similarly responds differentially to a limited number of odorant epitopes (Buck and Axel, 1991). Hence, the discriminatory code of the olfactory system cannot be based upon detecting the activity of only a single population of olfactory receptor neurons. It must involve recognizing the pattern of activity elicited by a particular odorant when it interacts with all of the complementary epitope-specific odorant receptors across the entire neuroepithelium. The topographical distribution of a particular odorant receptor on the neuroepithelial sheet could underlie this pattern generation. Although odorant induced activity across the neuroepithelium seems to possess regional variability (Thommesen and Doving, 1976; Mackay-Sim et al., 1982; Edwards et al., 1988), anatomical studies fail to demonstrate clustering of odorant receptor-specific olfactory receptor neurons. Instead, olfactory receptor neurons which express the same putative odorant receptor are randomly distributed throughout a specific zone of the epithelium, interspersed among olfactory receptor neurons expressing different putative odorant receptors of the same gene sub-family (Ressler et al., 1993). This interspersion of diverse odorant receptors within an epithelial zone precludes equating the activity within a zone with the activation of a particular odorant receptor. Clearly the topographical distribution of olfactory receptor neurons in the neuroepithelium cannot establish an odorant-specific pattern of activity for odorant discrimination.

Many studies have demonstrated that the primary afferents to the olfactory bulb target specific regions and establish a topography corresponding to the neuroepithelium (Greer et al., 1981; Stewart and Penderson, 1987). Just as the expression of a particular odorant sub-family seems to be restricted to a

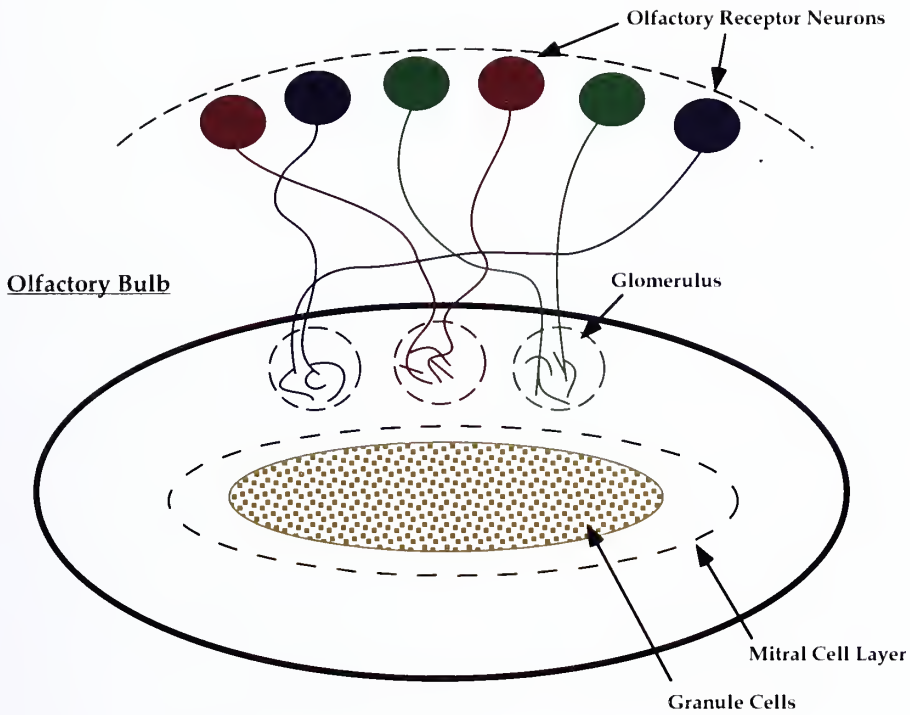
discrete epithelial zone, the axons from these neurons project to a particular region of the olfactory bulb. It thus seems apparent that the population of olfactory receptor neurons which express the same sub-family of putative odorant receptors, and are thus responsive to the same class of odorants, project to the same region of the olfactory bulb (Stewart and Pederson, 1987). In addition, it appears that functionally specific (Lancet et al., 1992) and odorant receptor-specific (Vassar et al., 1994) olfactory receptor neurons fasciculate during their course through the olfactory nerve layer suggesting consensual targeting. Odorant receptor-specific olfactory receptor neuron axons have also been demonstrated to converge upon particular glomeruli within the bulbar region defined by their sub-family (Vassar et al., 1994) (Figure 7).

This targeting and convergence of functionally specific primary afferents onto individual glomeruli would result in a singularly odorant-specific pattern of glomerular activation which could represent the discriminatory code employed by the olfactory system. Such an odortopic map is supported by a wealth of physiological evidence. Recording from single glomeruli, Leveteau and MacLeod (1966) were able to demonstrate differential responses in the same glomerulus to different odorants suggesting an ordering of its sensitivity to different odorants. Autoradiography with 2-deoxyglucose (Stewart et al., 1979; Jourdon et al., 1980) and high-resolution 2-deoxyglucose (Lancet et al., 1982) confirmed this differential response characteristic of individual glomeruli and also demonstrated unique laminar patterns of activity across the glomerular layer in response to different odorants. In addition, Buonviso and Chaput (1990) have revealed that mitral and tufted cells whose apical dendrites arborize within the same glomerulus more often respond similarly to the

Figure 7:

Schematic Diagram Depicting the Projection of Primary Olfactory Afferents to the Olfactory Bulb. Functional and anatomical studies indicate that primary afferent fibers from the neuroepithelium project to the olfactory bulb in a grossly topographical manner, such that activity in one region of the neuroepithelium can be correlated with activity in a particular region of the olfactory bulb. The interspersion of diverse olfactory receptors within any one region of the neuroepithelium prevents equating this regional activity with the activation of any single receptor type. Recent evidence suggests that the axons of odorant receptor-specific olfactory receptor neurons converge on specific glomeruli in the olfactory bulb (red to red; blue to blue; and green to green), thus establishing activity in a limited region of the olfactory bulb in response to the presentation of a particular odorant. The discriminatory code employed by the olfactory system, postulated to involve the distributed activity across the glomerular layer, would certainly depend on this specificity of projections. See text for further details.

Olfactory Neuroepithelium



presentation of a particular odorant.

The discriminatory code employed by the olfactory system appears to encompass the following model: the activation of a unique set of olfactory receptor neurons by a specific odorant, mediated by the association of odorant epitopes with their complementary odorant receptors on the surface of the olfactory receptor neurons, produces a unique pattern of activity across the glomerular layer of the olfactory bulb specific to the offending stimulus, and thus eliciting the particular subjective perception of that odorant. Therefore, the activity of a single olfactory bulb glomerulus represents the fundamental unit of this code. Further clarification of olfactory discrimination would seem to necessitate a more detailed understanding of the organization of the olfactory bulb glomerulus, which presently is lacking. The topological distribution of primary olfactory afferents within the olfactory bulb glomerulus as well as the degree to which individual primary afferent fibers target specific post-synaptic populations, versus the degree to which they interact homogeneously, remains to be elucidated. In Golgi-impregnated preparations, primary olfactory afferents have been demonstrated to arborize within restricted regions of the glomerulus rather than distributing diffusely throughout the glomerular neuropil (Hálasz and Greer, 1993). This suggests a degree of sub-compartmentalization within the glomerulus which may represent the segregation of intraglomerular circuits and could contribute to the discriminatory code employed by the olfactory system.

In the present study, the topological distribution of individual DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) labeled olfactory receptor neuron axons and fascicles, as they approach, penetrate, and arborize within individual glomeruli, are assessed by confocal microscopy. The hypothesis suggested by the Golgi-impregnated samples, that the

olfactory bulb glomerulus possesses a subcompartmental organization, is confirmed and further defined as a fascicle-specific organization by this technique. Primary afferents from the olfactory neuroepithelium seem to fasciculate quite far from their target glomerulus and, once in its confines, arborize within a limited area of the peripheral shell of the glomerulus. Additionally, fibers from different fascicles do not appear to overlap in their distribution, but instead remain fairly well segregated except over the borders of their fascicle-specific domains. It is postulated that these compartments of the glomerulus represent sites of independent processing of parallel pathways from the neuroepithelium to the central nervous system, which could signify: (1) the targeting of a discrete class of olfactory receptor neuron axons for a particular population of post-synaptic, second order neurons or (2) the delineation of odorant-specific domains within the olfactory bulb glomerulus, further defining the discriminatory code employed by the olfactory system.

STATEMENT OF PURPOSE

Recognizing the essential role of the olfactory bulb glomerulus as the fundamental unit of the discriminatory code employed by the olfactory system, it is necessary to understand the anatomy of those processes contributing to its complex neuropil, especially that of the primary afferent projections from the neuroepithelium. Although the Golgi-impregnation technique employed in previous studies of the topology of olfactory receptor neuron axons in olfactory bulb glomeruli affords visualization of the complete architecture of individual fibers, its capricious staining characteristics and reliance on light microscopy limit the possible analysis. A more detailed study of the topological distribution of single olfactory receptor neuron axons and fascicles as they approach, penetrate, and arborize within individual glomeruli of the olfactory bulb is possible with DiI staining and confocal microscopy. This would further both an understanding of the organization of the olfactory bulb glomerulus and how the olfactory bulb glomerulus precisely establishes the discriminatory code employed by the olfactory system. **The purpose of this study, therefore, is: (1) to expand on the results from previous Golgi-impregnation studies by performing a confocal microscopic analysis of the topological distribution of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) labeled primary olfactory afferents in olfactory bulb glomeruli of the rat; and (2) to test the hypothesis that the distribution of primary olfactory afferents contributes to a subcompartmental organization within the glomerulus.**

MATERIALS AND METHODS

Eleven two week old Sprague-Dawley rats were anesthetized with intraperitoneal injections of pentobarbital (0.2ml of a 50mg/ml solution) and allowed to develop complete loss of muscular tone prior to the opening of a midline, ventral thoracolaparotomy incision, from the base of the neck to the mid-pelvis. Lateral reflection of the ribs and anterior abdominal wall was then performed. This permitted both manipulation of the heart and simultaneous observation of hepatic and mesenteric structures during the subsequent perfusion.

After manually inserting the sharpened end (cut on the bias) of a length of polyethylene tubing (1.27mm external diameter/0.86mm internal diameter) into the left ventricle and incising the right atrium at its auricle, room temperature 0.9% sodium chloride was perfused at a rate of 4ml/minute, by a micro-dialysis pump. Perfusion was continued until visualization of hepatic and mesenteric bed paling and then for an additional 2 minutes. This perfusion with saline served only to clear the vascular system. Throughout this time, clots were removed from the site of blood egress at the right atrium to allow for uninterrupted flow. Additionally, the animal's neck was maintained with a linear relationship to its thorax to assure an unobstructed cerebral perfusion.

The perfusion apparatus was then removed, and the animals were placed on their ventral surface. Initially the skin was cut away and stripped from the head in order to visualize the intracranial structures through the thin skull. Subsequent decapitation with a straight edge razor blade, at the level of the cerebellum, was followed by removal of the superior aspect of the

skull. After separating it from the remaining skull with scissors, it could be easily dissected free from the underlying brain and removed. At this point, the intact head was carefully placed in 5°C 4% paraformaldehyde in 0.1M phosphate buffer for 5 minutes. This procedure stiffened the surface of the brain and greatly facilitated further dissection. Olfactory bulbs with intact forebrains were then dissected free from the surrounding bone and cranial nerves with Rongeurs and micro-scissors respectively and carefully placed in 5°C 4% paraformaldehyde. Complete fixation was achieved by immersion over the succeeding 5-16 (μ=10.2) days at room temperature. All remaining body parts were disposed of as per protocol.

It is important to emphasize the use of immersion fixation, as the perfusion of fixative was intentionally omitted. Preliminary studies revealed a disturbing correlation between paraformaldehyde perfusion and the stability of DiI in labeled processes. Although labeled processes could be identified and traced immediately after sectioning, there appeared to be a temporally related degradation in the specificity of this label. A useless haze was all that typically remained after as little as twelve to twenty-four hours at 5°C. This destabilization was not observed in those specimen which underwent immersion fixation, as repeated analysis was possible for weeks after sectioning. The nature of the correlation between perfusion of fixative and DiI destabilization was not investigated, however the extent of fixation does not appear to be a factor. Specimens prepared by either technique appeared to be equally stiff.

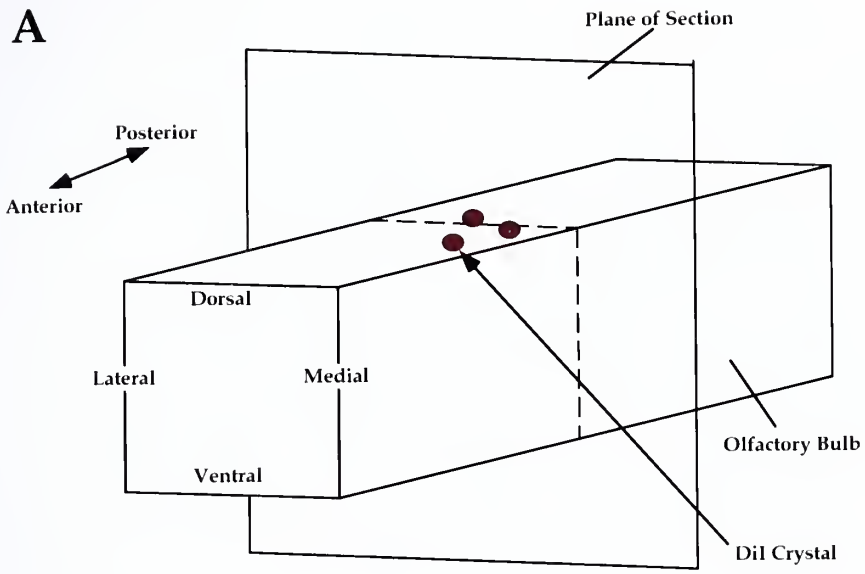
Following fixation, each specimen was divided in the mid-sagittal plane, thereby separating the two olfactory bulbs for individualized processing. Each olfactory bulb was subsequently implanted with multiple, typically 2-4, DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine

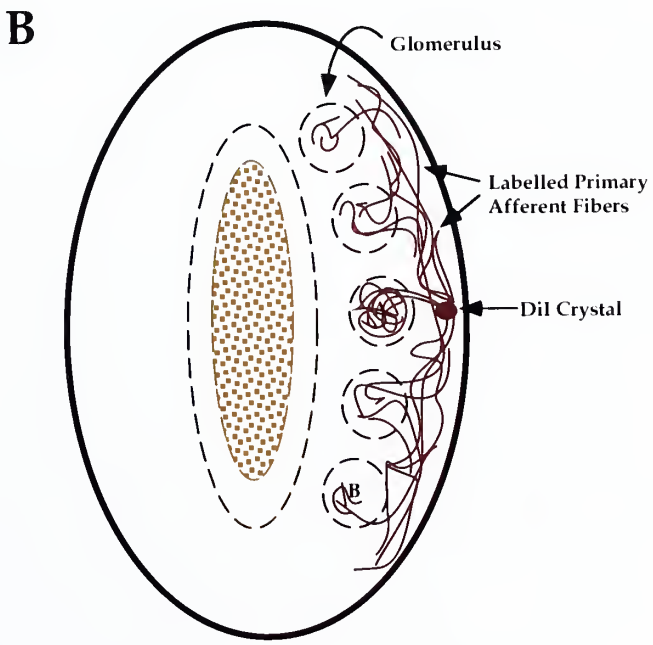
perchlorate; molecular weight 936 daltons) (Molecular Probes Catalog #D-282) crystals. Although their precise placement varied, individual crystals, usually three, were routinely clustered along the superior and medial edge of the olfactory bulb with a spacing of approximately 2mm (Figure 8A). Crystalline DiI was initially examined under a stereo-microscope and pulverized with a spatula to achieve a crystal size of approximately 250μm in diameter. Implantation involved puncturing small openings through the pia mater with the closed end of a Dumont #5 micro-forceps, manually placing individual DiI crystals over these openings, and pushing them into the most superficial layer of the olfactory bulb, the olfactory nerve layer. Precise placement exclusively within this layer was not essential to the labeling of primary olfactory afferent fibers, however deeper penetrations which allowed for labeling of principal and interneuron cell processes (mitral, tufted, and periglomerular cell dendrites) hindered the analysis of some glomeruli. To prevent any possible movement of the DiI crystals during the long transport period, a drop of warmed, free-flowing 5% agar was placed onto each implantation site and allowed to harden. DiI implanted olfactory bulbs were then returned to fresh, room temperature 4% paraformaldehyde, light protected, and maintained at room temperature (20-22°C) for an incubation period of 1-8 (μ=3.5) weeks.

During this time, individual olfactory bulbs were inspected for evidence of DiI transport. Those with extensive blushing around the site of crystal injection (approximately 2-3mm) were deemed complete and processed for analysis. The olfactory bulbs were rinsed in 0.1M phosphate buffer, embedded in 5% agar on a tissue stage, and cut transversely at 100μm with a vibratome. Sections were obtained from the rostral tip of the olfactory bulb to the level of the anterior olfactory nucleus. Individual tissue sections

Figure 8:

Schematic Diagram Illustrating the Implantation of DiI crystals into the Olfactory Nerve Layer of the Olfactory Bulb (A) and a Magnified Tissue Section Through the Site of Crystal Placement (B). (A) DiI crystals, approximately 250μm in diameter were manually inserted into the most superficial layer of the olfactory bulb to label the axons of olfactory receptor neurons. Although precise positioning varied across specimen, three individual crystals were generally implanted along the superior and medial edge of the olfactory bulb, with a spacing of approximately 1-2mm. **(B)** Olfactory bulbs were embedded in 5% agar on a tissue stage and cut transversely at 100μm with a vibratome. Individual sections were visualized on an Olympus IMT2 microscope equipped with a 40X objective and a fluorescein filter set. A representative section through the site of DiI crystal placement is illustrated here. The olfactory nerve layer and glomeruli in proximity to the DiI crystal were generally too well stained to permit analysis of the topological distribution of individual fibers, even with the confocal microscope. The glomerulus marked A, for example, contains a mesh of labeled processes which obscure the course of single fibers. Those glomeruli containing fewer stained processes, on account of their greater distance from the DiI crystal, as in the glomerulus marked B, often demonstrated the complete arborization pattern of single olfactory receptor cell axons and fascicles. These glomeruli were selected for further analysis with confocal optical sectioning.


A
Plane of Section
Posterior
Anterior
Dorsal
Lateral
Medial
Ventral
Olfactory Bulb
DiI Crystal


B
Glomerulus
Labelled Primary
Afferent Fibers
DiI Crystal

were placed in 0.1M phosphate buffer for removal of the surrounding agar. With a small, fine bristle brush, tissue sections were then float mounted onto gelatin coated slides, covered with 2-3 drops of 0.1M phosphate buffer, and coverslipped. To prevent evaporation of the phosphate buffer and desiccation of the tissue, the edges of the coverslip were sealed with nail polish. Visualization with an Olympus IMT2 microscope equipped with a 40X objective and a fluorescein filter-set was then performed, using both epi-fluorescence and a BioRad 600 Krypton-Argon confocal laser. Following analysis, the individual slides were light protected and stored at 5°C for future inspection and comparison.

Using epi-fluorescent light microscopy, individual glomeruli were selected for confocal analysis (Figure 8B). Selection criteria principally involved identifying those glomeruli in which the complete arborization pattern of single olfactory receptor cell axons could be discerned (B in Figure 8B). Hence, those glomeruli in which a mesh of labeled processes obscured the course of single fibers were typically rejected (A in Figure 8B). Glomeruli only partially confined to a single 100μm section were categorically rejected. Additional criteria involved the ability to trace these fibers from their glomerular arbors proximally into the olfactory nerve layer and to identify their fascicles of origin. After selecting a glomerulus for further investigation, the microscope was focused at an approximate mid-position within the glomerulus and confocal analysis was begun.

Initially it was essential to determine the Z-axis dimension of the glomerulus. This would define the size of the glomerulus, confirm that an entire glomerulus was being imaged, and set the limits of optical sectioning. By adjusting the Z-axis focus of the confocal laser and inspecting the confocal image at each level, the upper and lower limits of a glomerulus were easily

identified. Typically, as one optically sectioned, the labeled processes would disappear before the appearance of the periglomerular network surrounding a single glomerulus. The limits of optical sectioning were therefore set at the first and last image containing labeled processes and was carried out to the tenth of a micron step. The confocal Z-axis was then set at the exact mid-position of the glomerulus for refining of the confocal image.

Using photon counting, the amount of signal and background noise could be graphically displayed and adjusted to better define the labeled processes of interest. The methodology involved adjusting the aperture, black level, and gain and then eliminating the large photon peaks, which presumably represented the background haze, from the generation of the image. This produced clear images in which individual axons could be easily identified and traced. Optical sections, 0.5-1.0μm thick, were then captured in series using the pre-determined Z-axis limits of the glomerulus. The typical glomerulus encompassed a series of 20-40 sections which were stored as digitized images for further analysis.

Those sections encompassing stained processes of interest were reconstructed to obtain a detailed 2-dimensional image. To further our analysis, 3-dimensional reconstructions, employing an optical shift of 0.20-2.0, were also produced. Reconstructed arbors were assessed for number of bifurcations, topological distribution within individual glomeruli, and evidence of colocalization of labeled axons from the same fascicle. For display and publication purposes, optical images were reproduced in two ways: 1) 35mm photos were obtained directly off of the computer monitor display or 2) digitized images were converted into Macintosh format and printed with an Adobe Photoshop software package on a Codonics 1600 color printer.

Please note: the production of all materials and performance of all procedures described above were conducted independently by the student after receiving preliminary instruction and guidance.

RESULTS

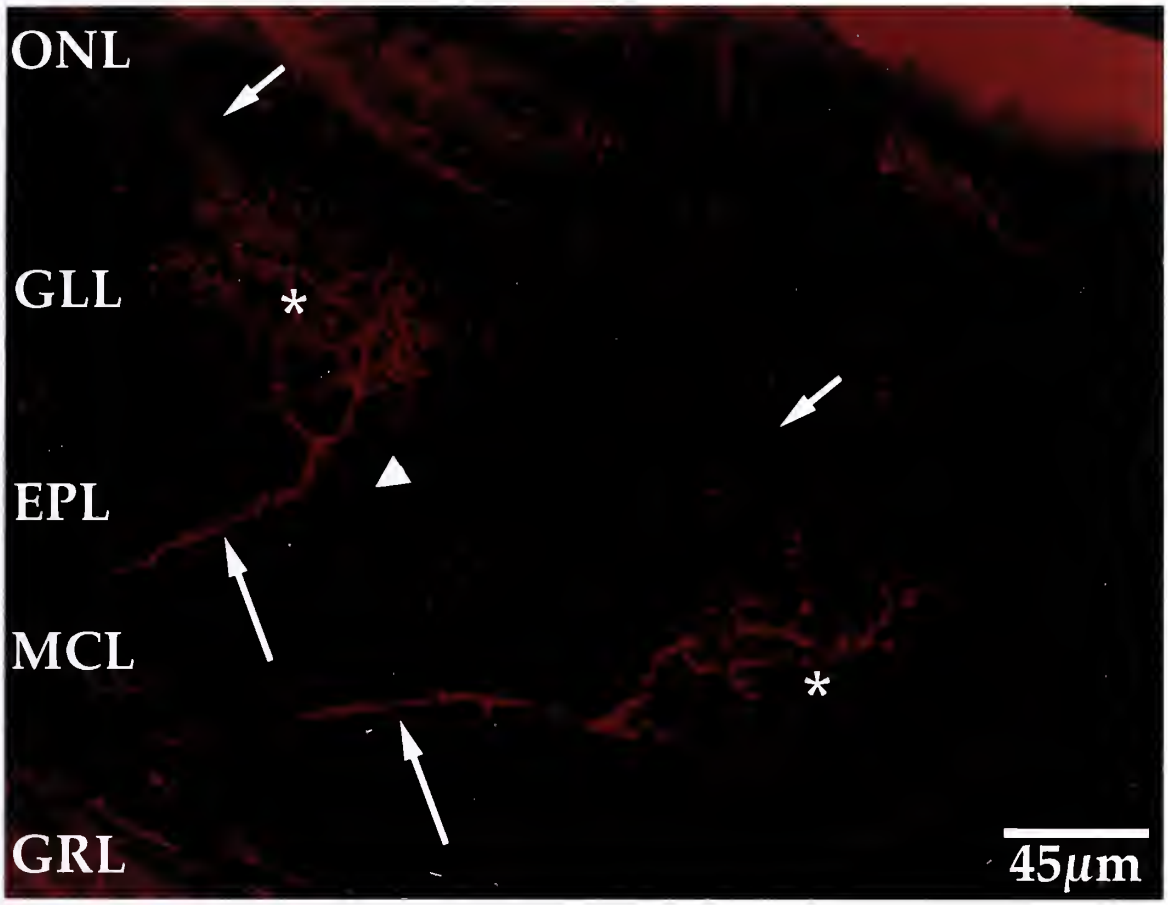
General Anatomy of the Olfactory Bulb

The distinctive laminar architecture of the olfactory bulb is evident in cresyl-violet stained sections where the soma of its individual neuronal elements are labeled (Figure 3). From superficial to deep, the five symmetric layers are clearly demonstrated: olfactory nerve layer (ONL), glomerular layer (GLL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), and granule cell layer (GRL). The most striking feature is surely the round, 40-110μm diameter regions of neuropil within the glomerular layer, the olfactory bulb glomeruli (asterisks and a). Forming calyx-like structures, the manner in which periglomerular cell soma surround and demarcate individual glomeruli is evident in Figure 3 (Glomerulus a). These regions of the olfactory bulb represent the site of the first synapse in odorant processing, where the terminal arbors of olfactory receptor neurons axons, the apical dendrites of mitral and tufted cells, and the dendritic processes of periglomerular cells overlap.

The simultaneous labeling of multiple intrabulbar elements by DiI often yielded images in which the laminar organization of the olfactory bulb was similarly apparent, but in addition, in which the cytological architecture of its individual neuronal elements are demonstrated (Figure 9). Single primary afferent axons (short arrows) are identified and can be traced both as they course caudally through the olfactory nerve layer (ONL) and as they penetrate deeper into the glomerular layer (GLL) to ramify and terminate within individual glomeruli (asterisks). Careful analysis of these labeled

Figure 9:

Confocal Microscopy of DiI Labeled Processes in the Olfactory Bulb. Although DiI crystals were implanted into the olfactory nerve layer to label the axons of olfactory receptor neurons, occasional deeper penetrations and free diffusion from the olfactory nerve layer often yielded serendipitous staining of all bulbar elements. In this example, the laminar organization of the olfactory bulb is apparent. Individual primary afferent fibers (short arrows) can be traced from the olfactory nerve layer (ONL) into individual glomeruli (asterisks) where their complex terminal arborizations are evident. The colocalization of the terminal ramifications of apical dendrites of mitral and tufted cells (long arrows) within glomeruli is also demonstrated. Within the glomerular layer (GLL), a periglomerular somata (arrowhead) can be identified adjacent to a region of glomerular neuropil. DiI clearly has the capacity to label all of the individual bulbar elements in a recognizable fashion. [ONL=olfactory nerve layer; GLL=glomerular layer; EPL=external plexiform layer; MCL=mitral cell layer; GRL=granule cell layer; long arrows=apical dendrites of mitral or tufted cells; short arrows; axons of olfactory receptor neurons; arrowhead=periglomerular cell somata; asterisks=glomeruli]


ONL
GLL
EPL
MCL
GRL
45μm

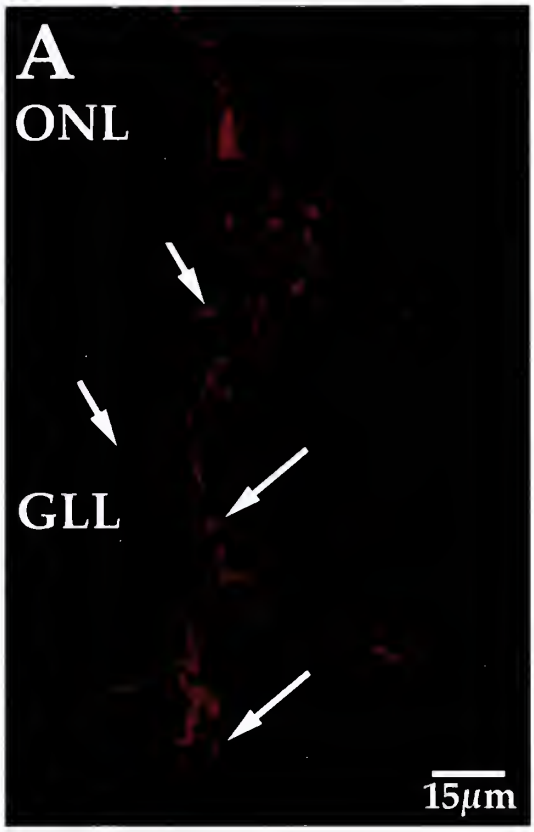
processes, both in this figure and in a three-dimensional reconstruction (not shown), failed to demonstrate any evidence of branching of olfactory receptor neuron axons prior to entering a glomerulus. Within the glomerulus, the terminal arbors of primary afferents appear to distribute in a complex pattern throughout the glomerular neuropil, measuring 40-110μm in this figure. Adjacent to one of these glomeruli, a periglomerular somata (arrowhead) can be recognized. Ascending through the external plexiform layer (EPL) and into these same glomeruli, the apical dendrites (long arrows) of mitral or tufted cells are also delineated. The two apical dendrites observed in this figure measure 4-5μm in diameter and follow a linear course toward individual glomerular targets, consistent with observations of previous investigators. Upon reaching the glomerulus, each apical dendrite appears to arborize in a stereotypic manner: the main dendrite branches into two trunks, each of which then ramifies to form a complex terminal arbor overlapping the terminal arbors of the axons of olfactory receptor neurons. Thus, this figure demonstrates the colocalization of the terminal processes of olfactory receptor neuron axons and mitral or tufted cell apical dendrites within single glomeruli. Elucidation of the topological features of these arbors is difficult secondary to the number of labeled processes in these glomeruli.

Architecture and Topological Distribution of Single Primary Afferent Fibers

The terminal arbors of individual olfactory receptor neuron axons were often singly labeled within a glomerulus. Representative examples are demonstrated in Figures 10 and 11. Single axons are observed to penetrate the glomerular neuropil and then branch into multiple intraglomerular axonal

Figure 10:

Confocal Microscopy of Single DiI Labeled Olfactory Receptor Neuron Axons. The variability with which the axons of olfactory receptor neurons arborize within olfactory bulb glomeruli is evident in these two figures. At high magnification, as in (A), individual axons are observed to branch into 2-6 collaterals when forming their terminal arbors. In more complex fibers, as in (B), the restricted domain with which an individual axon distributes its collaterals is demonstrated. The terminal arbors of individual olfactory receptor neurons do not appear diffusely distributed throughout the glomerular neuropil, but instead they remain confined within only a limited area of the glomerulus. Among the terminal arbors of these fibers, axonal varicosities (long arrows) and terminal boutons (short arrows), the sites of synaptic interactions, are demonstrated. These observations are consistent with those in Golgi-impregnated samples and confirms that DiI is staining the same population of fibers. Note: Glomerular borders are not visible in these highly magnified images. [ONL=olfactory nerve layer; GLL=glomerular layer; short arrows=terminal boutons; long arrows=axonal varicosities]


A
ONL
GLL
15μm

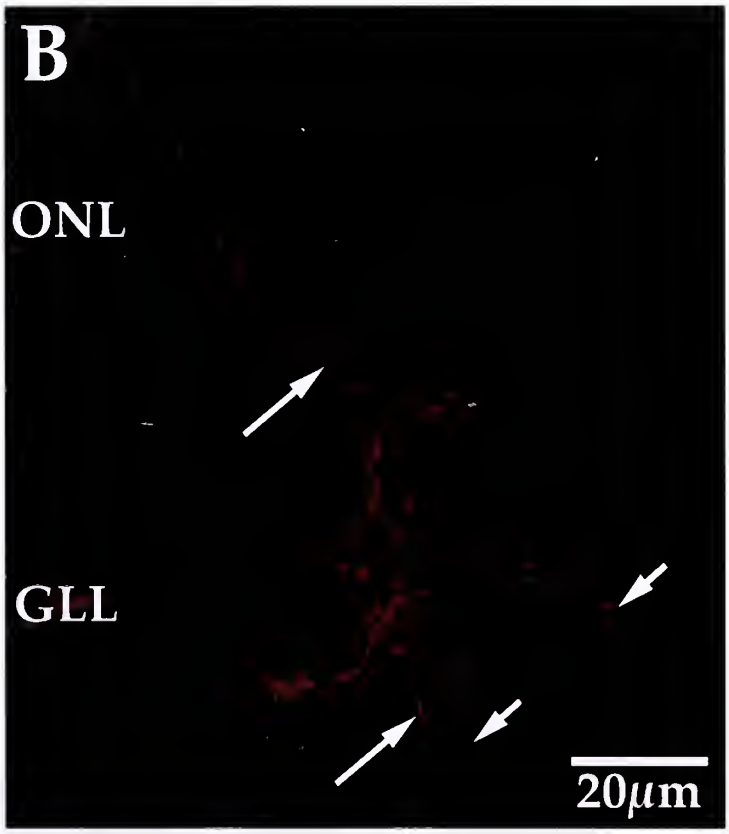
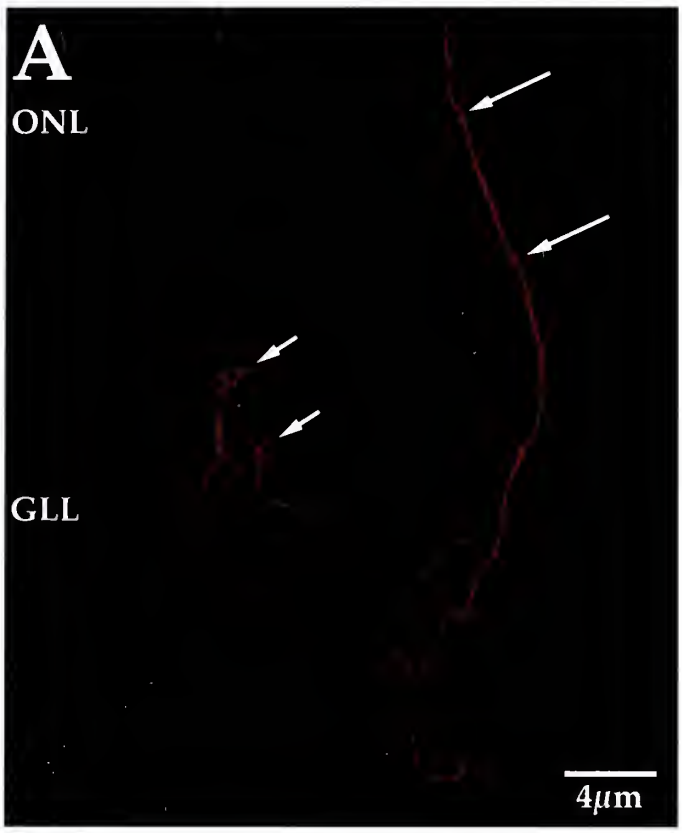
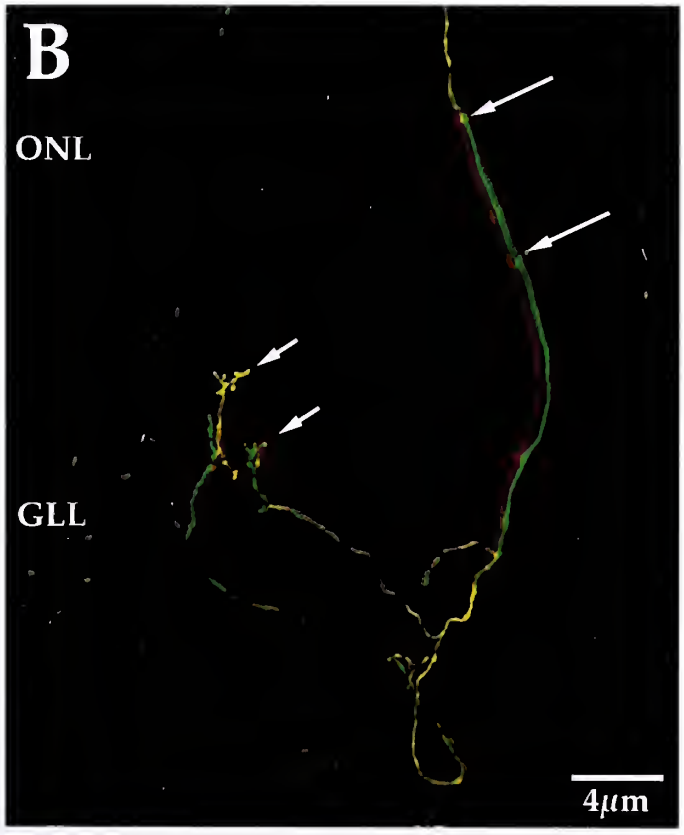

B
ONL
GLL
20μm

Figure 11:

Confocal Microscopy of a Single DiI Labeled Olfactory Receptor Neuron Axon: two-dimensional optical reconstruction (A) and three-dimensional optical reconstruction (B). The tortuous course followed by this axon as it arborizes in an olfactory bulb glomerulus suggests specificity of targeting for post-synaptic elements. As in other individual olfactory receptor neuron axons studied, both axonal varicosities (long arrows) and terminal boutons (short arrows) are demonstrated. Additionally, the limited area of the glomerulus occupied by the terminal arbor of this fiber is consistent with previous observations in Golgi-impregnated samples. Note: Glomerular borders are not visible in these highly magnified images. [ONL=olfactory nerve layer; GLL=glomerular layer; short arrows=terminal boutons; long arrows=axonal varicosities]


A
ONL
GLL
4μm


B
ONL
GLL
4μm

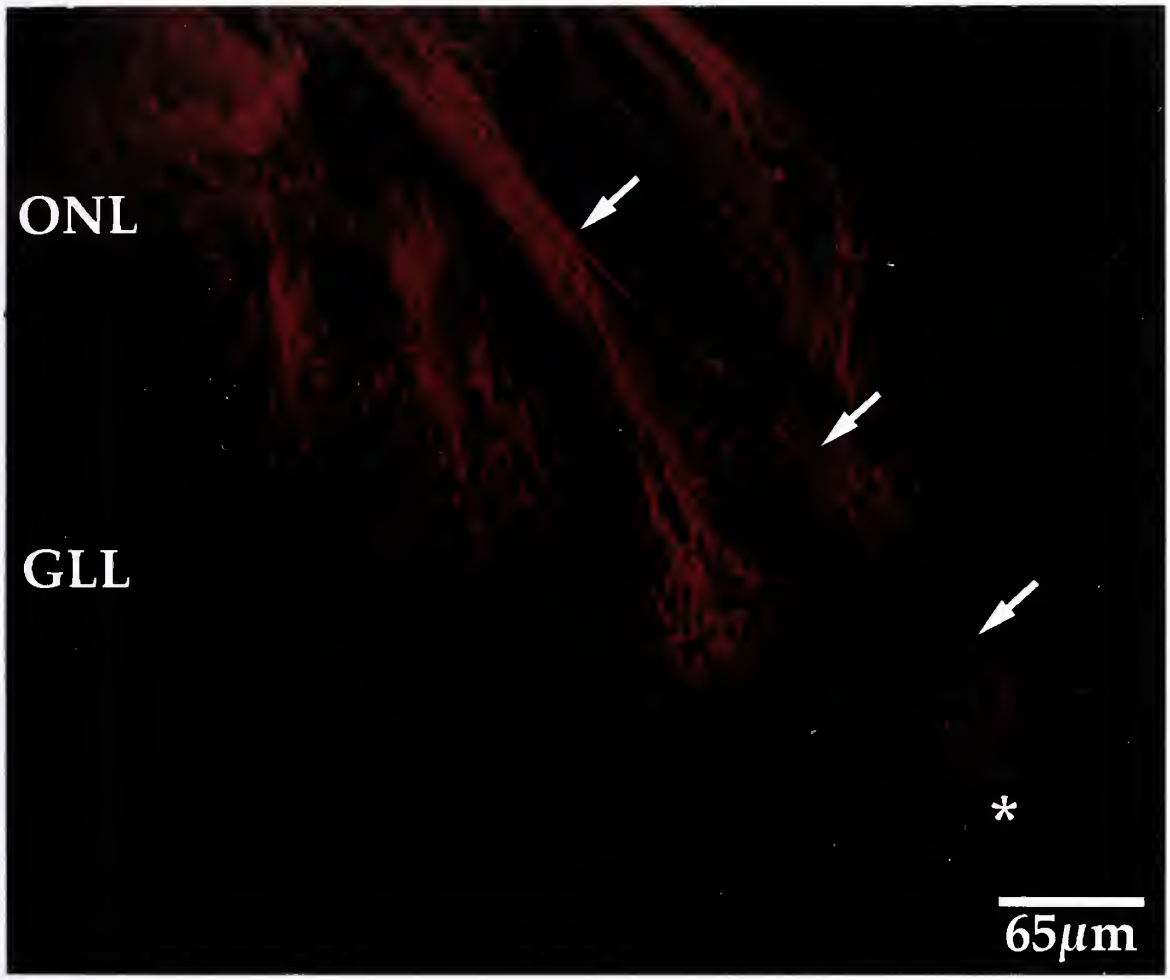
collaterals, ranging from 2-6. En passant varicosities (long arrows) and terminal boutons (short arrows), the sites of synaptic interactions, are demonstrated among these arbors. Possessing varying degrees of complexity and tortuousity, the fibers depicted in these figures form heterogeneous ramification patterns. While the axon in Figure 10A appears to innervate a columnar region of the glomerular neuropil, the arbors of the axon in Figure 10B seem to form a sphere. In Figure 11, an example of the tortuous course followed by some primary afferent fibers to their targets is demonstrated. This fiber literally turns back upon itself to reach a specific post-synaptic element. Yet irrespective of the pattern produced by their terminal arbors, individual axons do not appear to distribute throughout the glomerular neuropil. It is evident that the region of the glomerulus occupied by the olfactory receptor neuron axons depicted in these figures represents only a small proportion of the total glomerular volume. This is consistent with observations in Golgi-impregnated samples (Hálasz and Greer, 1993).

Fascicles of Olfactory Receptor Neuron Axons Target Specific Glomeruli

Fascicles of olfactory receptor neuron axons can be identified in the olfactory nerve layer (ONL), traced through their course into the glomerular layer (GLL), and observed to ramify within glomeruli (Figure 12). With a diameter of 3-40μm, discrete fascicles are apparent and can be traced proximally into the olfactory nerve layer for quite some distance from the glomeruli in which they ramify. The fascicle indicated by arrows, for example, is clearly demarcated more than 250μm away from the glomerulus in which it terminates (asterisk). In addition, there is a conspicuous absence

Figure 12:

Confocal Microscopy of Fascicles of DiI Labeled Olfactory Receptor Neuron Axons. Individual olfactory receptor neuron axons destined to arborize in the same glomerulus seem to fasciculate quite far from their destination and then travel the remaining distance together. The glomerulus marked with an asterisk in this figure is clearly targeted by axons that fasciculate more than 250μm away (arrows). Other examples of fascicular targeting for specific glomeruli are evident in this figure. These fascicles may correspond to the functional (Lancet et al., 1982) or odorant receptor-specific (Vassar et al., 1994) fascicles which other investigators have demonstrated. Once within the confines of the glomerulus, the axons from these fascicle do not arborize throughout the glomerular neuropil, but seem to remain restricted within a seemingly fascicle-specific sub-compartment of the glomerulus. The borders of the marked glomerulus (asterisk) in this figure are evident but do not appear to be filled with the processes from the one visible fascicle. A relatively large proportion of the glomerulus remains without DiI label. [ONL=olfactory nerve layer; GLL=glomerular layer; arrows=fascicle of olfactory receptor neuron axons; asterisk=glomerulus]


ONL
GLL
*
65μm

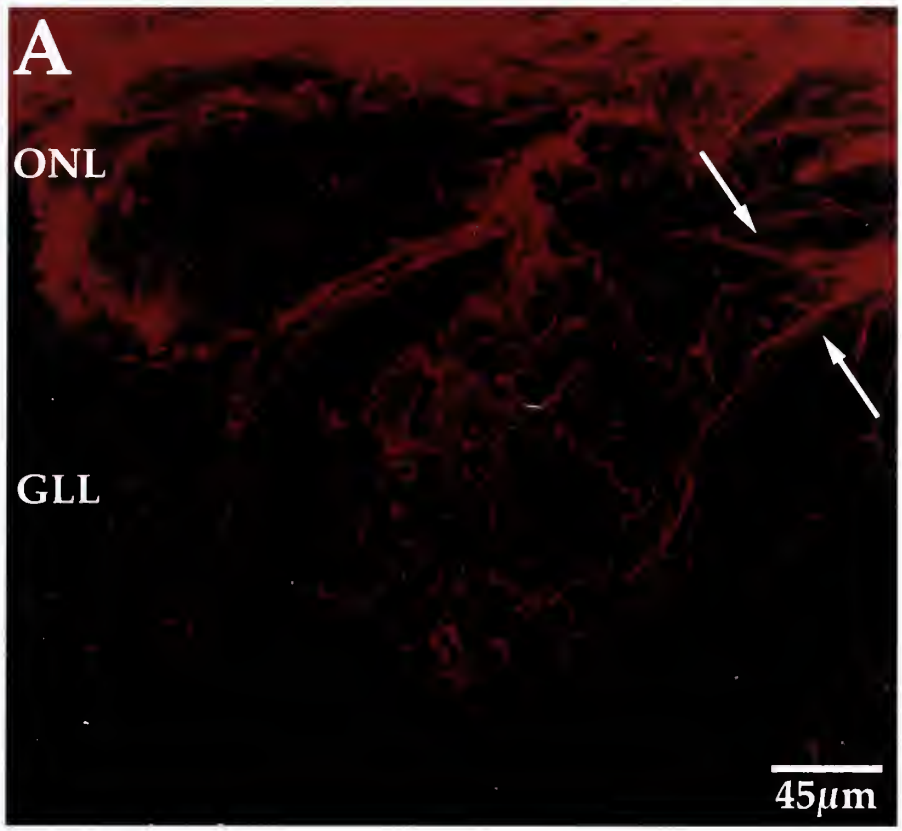
of branching by this and the other fascicles demonstrated. Although the indicated fascicle (arrows) may appear to branch from a larger bundle, three-dimensional reconstructions (not shown) confirm the existence of two independent fascicles. Single fascicles are not observed to collateralize and diffusely innervate multiple glomeruli, but instead they target specific glomeruli in which to form their terminal arbors. Within the glomerular neuropil, the terminal arbors of the axons from the indicated fascicle (arrows) overlap in a restricted region, rather than distributing throughout the glomerulus (asterisk). This topological distribution may represent the formation of fascicle-specific sub-compartments within the glomerulus.

Topological Distribution of Fascicles of Olfactory Receptor Neuron Axons

Most common among the glomeruli selected for confocal analysis were single glomeruli penetrated by two distinct fascicles (arrows in Figures 13-16). The terminal arbors of those axons from the same fascicle are observed to be colocalized within a limited region of the glomerulus, segregated from the terminal arbors of those axons from a different fascicle, thus forming fascicle-specific sub-compartments within the glomerular neuropil. Only minimally along the borders of these sub-compartments do the axons from different fascicles overlap. The two fascicles (arrows) in Figure 13 for example, arborize within distinctly separate regions (asterisks) of the glomerulus and interdigitate only where their individual compartments meet in the sparsely labeled center of the glomerulus. In addition, the terminal arbors within these sub-compartments appear to be concentrated in the peripheral shell of the glomerulus with less dense innervation of the glomerular core.

Figure 13:

Confocal Microscopy of an Olfactory Bulb Glomerulus Penetrated by Two Distinct Fascicles (arrows) of Olfactory Receptor Neuron Axons. In (A), the colocalization of arbors of olfactory receptor neuron axons from the same fascicle and the segregation of arbors of those axons from different fascicles are demonstrated. In (B), a higher magnification displays greater detail of this restricted distribution of axons within fascicle-specific sub-compartments (asterisks) of the glomerulus. Only minimally along the borders do the axons of these sub-compartments interdigitate. [ONL=olfactory nerve layer; GLL=glomerular layer; arrows=fascicles of olfactory receptor neuron axons; asterisks=fascicle-specific sub-compartments]


A
ONL
GLL
45μm

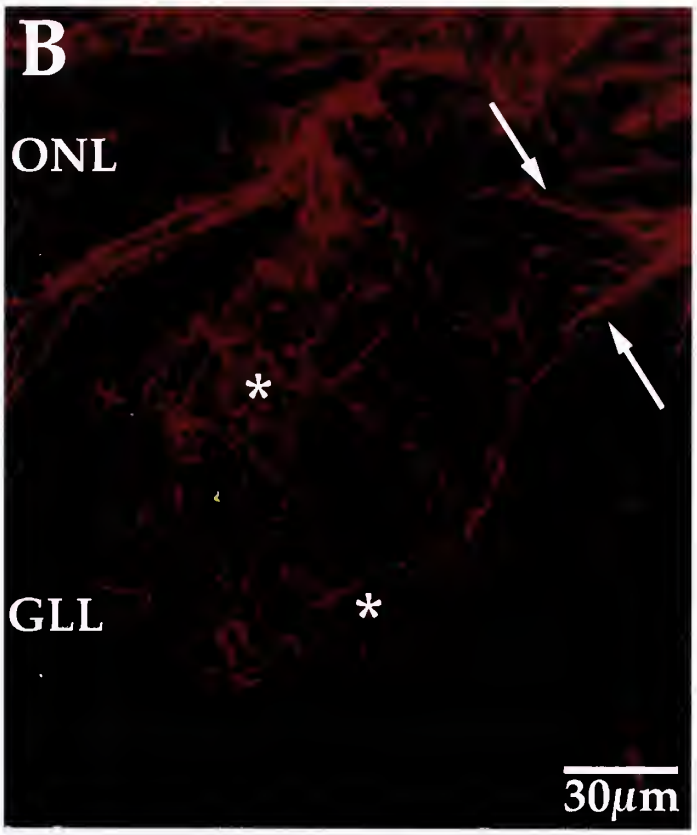
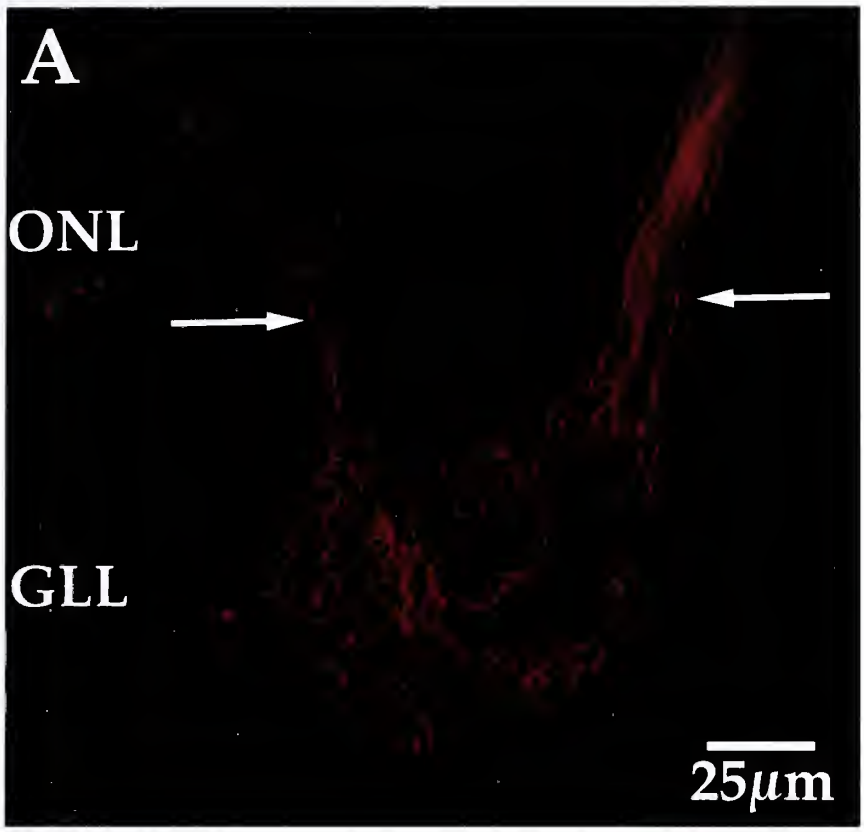

B
ONL
GLL
30μm

Figure 14:

Confocal Microscopy of an Olfactory Bulb Glomerulus Penetrated by Two Distinct Fascicles (arrows) of Olfactory Receptor Neuron Axons. In (A), the colocalization of arbors of olfactory receptor neuron axons from the same fascicle and the segregation of arbors of those axons from different fascicles are demonstrated. In (B), a higher magnification displays greater detail of this restricted distribution and suggests that, within these fascicle-specific sub-compartments, the arbors of olfactory receptor neuron axons seem to be concentrated in the peripheral shell of the glomerulus with less dense innervation of the glomerular core (asterisk). [ONL=olfactory nerve layer; GLL=glomerular layer; arrows=fascicles of olfactory receptor neurons; asterisk=core of glomerulus]


A
ONL
GLL
25μm

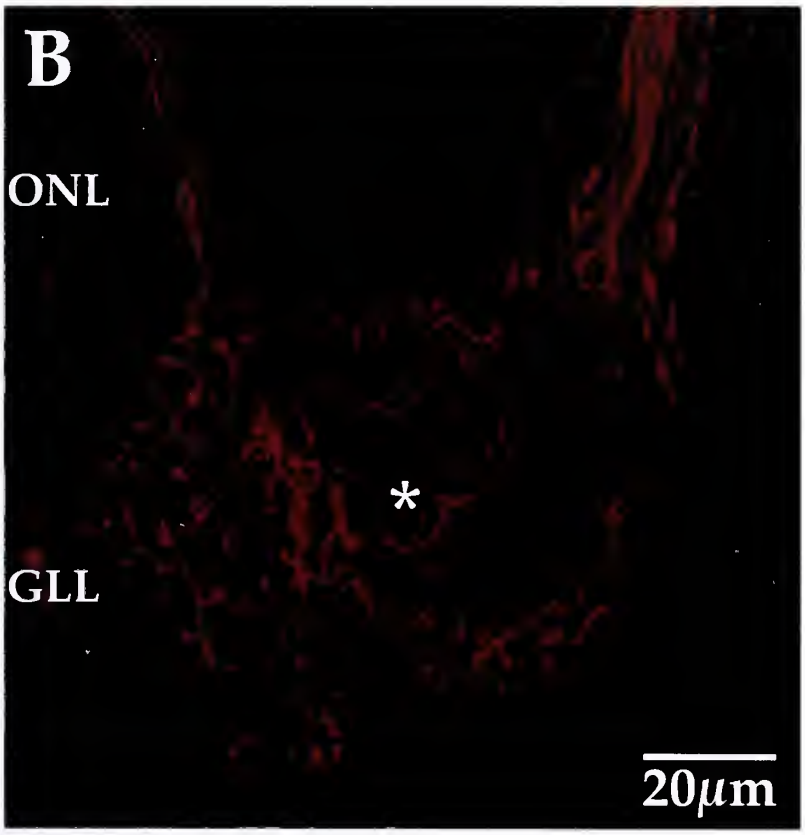
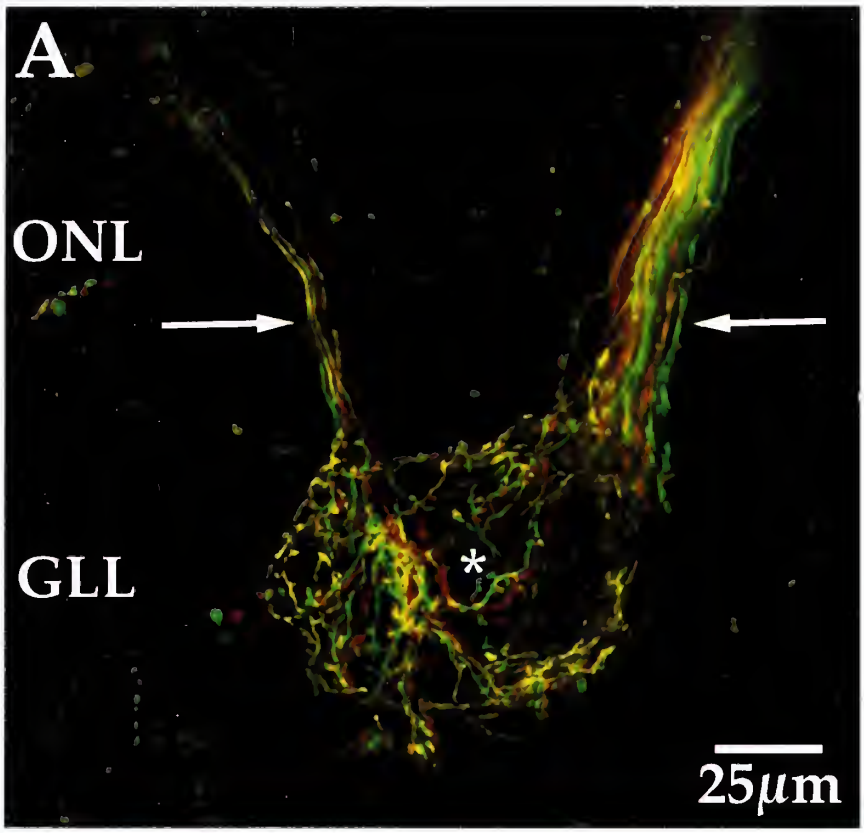
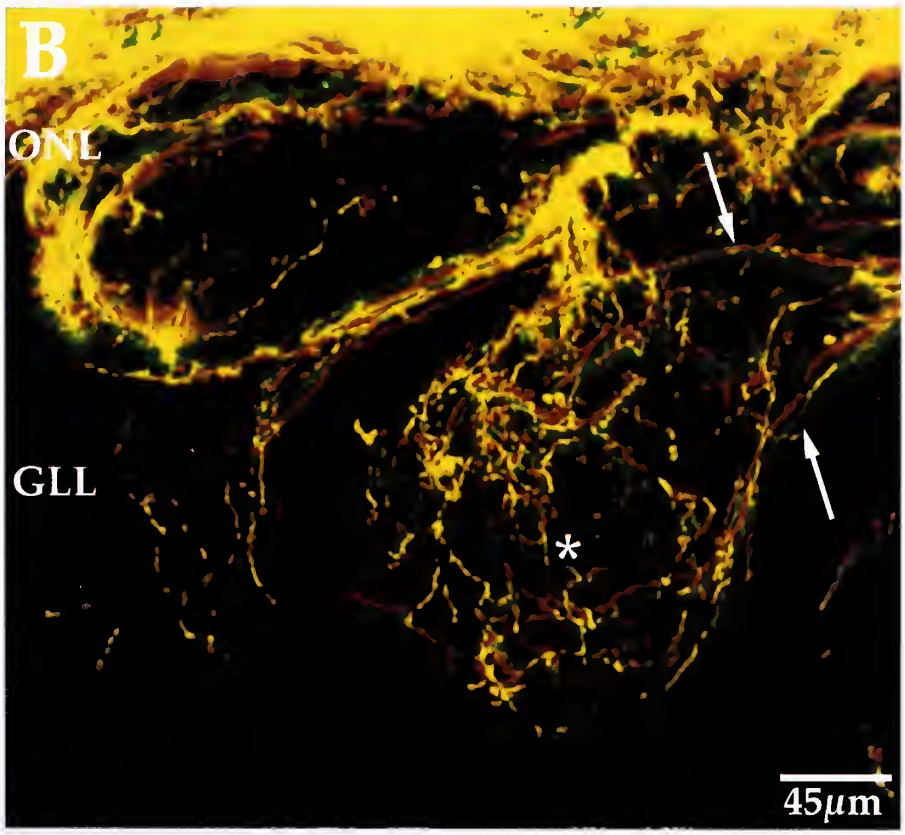

B
ONL
*
GLL
20μm

Figure 15:

Three-dimensional Confocal Optical Reconstructions of Glomeruli Penetrated by Two Distinct Fascicles of Olfactory Receptor Neurons (arrows): (A) corresponds to Figure 14A and (B) corresponds to Figure 13A. These images clearly demonstrate the segregation of the arbors of those axons from the same fascicle within a sub-compartment of the glomerulus. Interdigitation between the axons of different fascicles is only observed along the borders of their individual compartments. In addition, the axonal arbors do not appear to ramify throughout the glomerulus but, as demonstrated in these three-dimensional images, they appear to be concentrated in the peripheral shell of the glomerulus with few fibers penetrating into the glomerular core (asterisks). [ONL=olfactory nerve layer; GLL=glomerular layer; arrows=fascicles of olfactory receptor neurons; asterisks=glomerular core]


A
ONL
GLL
*
25μm


B
ONL
GLL
*
45μm

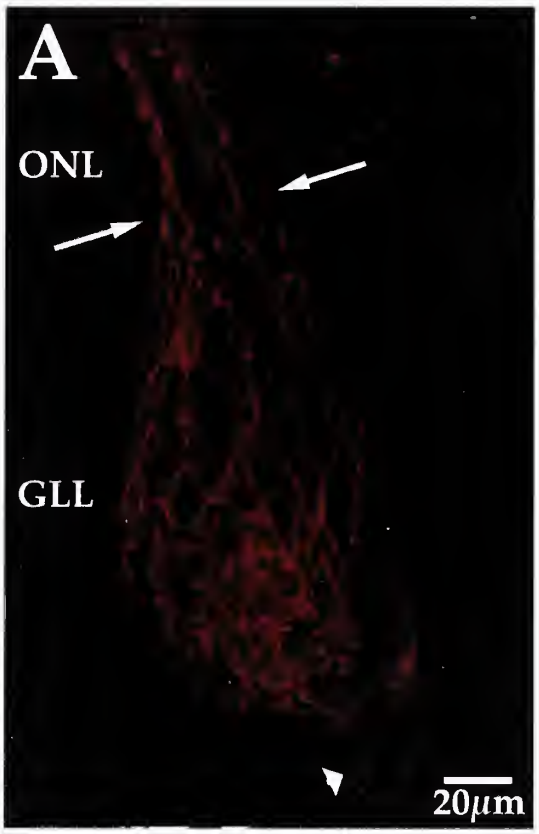
Although fascicle-specific sub-compartments are not as easily recognized in Figure 14, the terminal arbors of the olfactory receptor neuron axons innervating this glomerulus are observed to form a spherical shell about the periphery of glomerulus with the suggestion of an empty core (asterisk). Three-dimensional reconstructions of these glomeruli (Figure 15) confirm that the axons of olfactory receptor neurons distribute within fascicle-specific sub-compartments and concentrate their terminal arbors within the peripheral shell of the glomerulus.

Targeting by Olfactory Receptor Neuron Axons

Among the terminal arbors of olfactory receptor neuron axons, some individual fibers are observed to follow tortuous paths (Figure 11 and arrowheads in Figures 16 and 17) to their targets. In Figure 16, a small bundle of 2-3 axons (arrowhead) appears to proceed straight through the apparent edge of the glomerulus, only to complete a wide 180° turn, and enter a region of the glomerulus packed with the arbors of other olfactory receptor neuron axons. Other axons seem to execute obscure turns in order to re-establish a desired trajectory (Figure 11 and arrowhead in Figure 17). The two left-hand 90° turns performed by the axon in Figure 17 place it on the seemingly same course on which it started. Similarly, the single fiber labeled in figure 11 turns back upon itself to reach a proximally situated target. These observations suggest extreme specificity for post-synaptic targets by the terminals of olfactory receptor neuron axons.

Figure 16:

Confocal Microscopy of an Olfactory Bulb Glomerulus Penetrated by Two Distinct Fascicles (arrows) of Olfactory Receptor Neuron Axons: two-dimensional view (A) and three-dimensional reconstruction (B). These images lend further support to the proposition that the arbors of the axons of olfactory receptor neurons within olfactory bulb glomeruli form fascicle-specific sub-compartments confined mostly to the peripheral shell of the glomerulus. Within these sub-compartments, a presumable specificity of targeting by individual axons for post-synaptic processes is suggested by the tortuous and somewhat roundabout course followed by some of the axons studied (arrowhead). [ONL=olfactory nerve layer; GLL=glomerular layer; arrows=fascicles of axons of olfactory receptor neurons; arrowhead=olfactory receptor neuron axons]


A
ONL
GLL
20μm

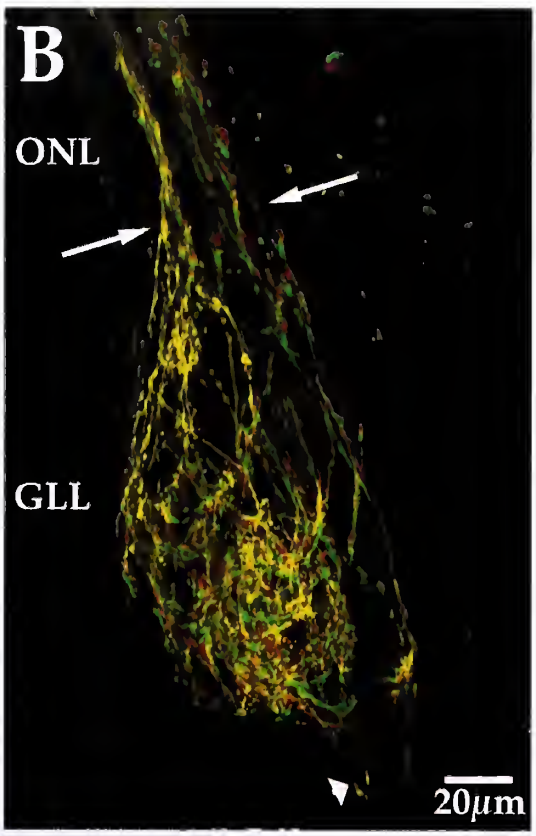
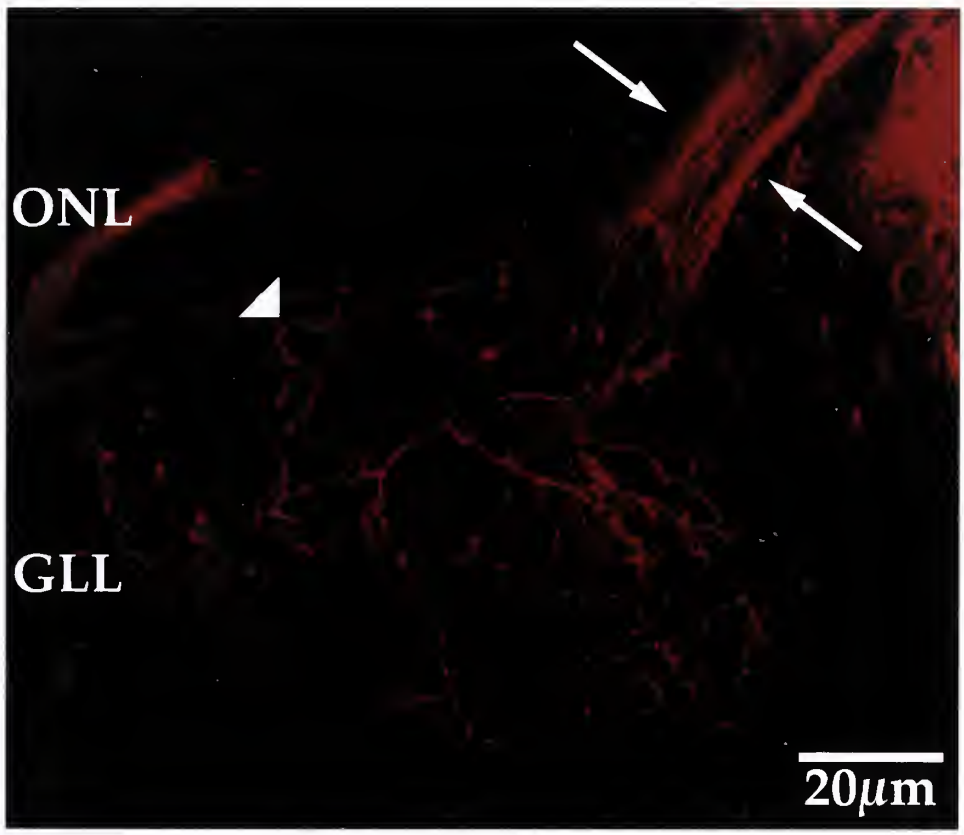

B
ONL
GLL
20μm

Figure 17:

Targeting by Primary Afferent Fibers in Olfactory Bulb Glomeruli. Although the topological distribution of the two fascicles ramifying in this glomerulus lacks the clarity of other images, the tortuous course (arrowhead) followed by an individual axon is demonstrated. This adds increasing support for a presumable specificity of targeting by individual olfactory receptor neuron axons for post-synaptic sites. [ONL=olfactory nerve layer; GLL=glomerular layer; arrows=fascicles of axons of olfactory receptor neurons; arrowhead=olfactory receptor neuron axon]


ONL
GLL
20μm

Principal and Periglomerular Elements

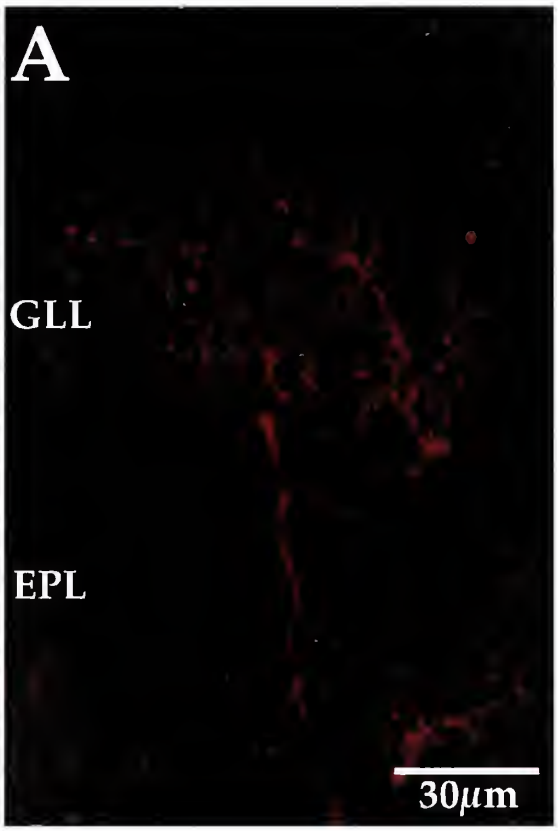
The free diffusion of DiI into deeper lamina of the olfactory bulb often labeled a diversity of neuronal elements, including but not limited to the apical dendrites of principal neurons (Figure 18) and periglomerular cells (Figure 19).

The apical dendrites of mitral and tufted cells are observed in their ascending course through the external plexiform layer (EPL) and in forming terminal arbors in individual glomeruli in the glomerular layer (GLL). As observed in Figure 9, the apical dendrites of principal neurons appear to ramify in a stereotypic pattern. While consistent with these previous observations, the higher magnification in this figure affords a more detailed analysis of this arborization pattern. The apical dendrite appears to successively branch in two, thus yielding four terminal limbs from which complex arbors expand radially throughout the glomerulus. Thus unlike the terminal arbors of olfactory receptor neuron axons which appear to be restricted to the peripheral shell of the glomerulus, the dendritic processes of mitral and tufted cells seem to occupy a central position in the glomerulus, filling its core, and spreading outward into the peripheral shell.

A periglomerular cell is depicted in Figure 19. Although its axon is not contained in this image, the complex dendritic processes (long arrows) and 5μm diameter somata (short arrow) characteristic of this cell type are apparent. Like the terminal arbors of the apical dendrites of mitral and tufted cells, the dendritic arbors of periglomerular cells appear to occupy a more central position within the glomerulus.

Figure 18:

Confocal Microscopy of DiI Labeled Terminal Arbors of Apical Dendrites of Principal Neurons of the Olfactory Bulb: (A) and (B). The apical dendrites of mitral and tufted cells appear to arborize in a stereotypic pattern. After successively branching in two, four terminal limbs ramify to form complex arbors which expand radially throughout the glomerulus. The terminal tufts of these processes thus occupy the central core of the glomerulus and radiate outward into the periphery. They fill that region of the glomerulus left vacant by the arbors of olfactory receptor neuron axons and overlap with the primary afferents mostly where they extend into the peripheral shell of the glomerulus. [GLL=glomerular layer; EPL=external plexiform layer]


A
GLL
EPL
30μm

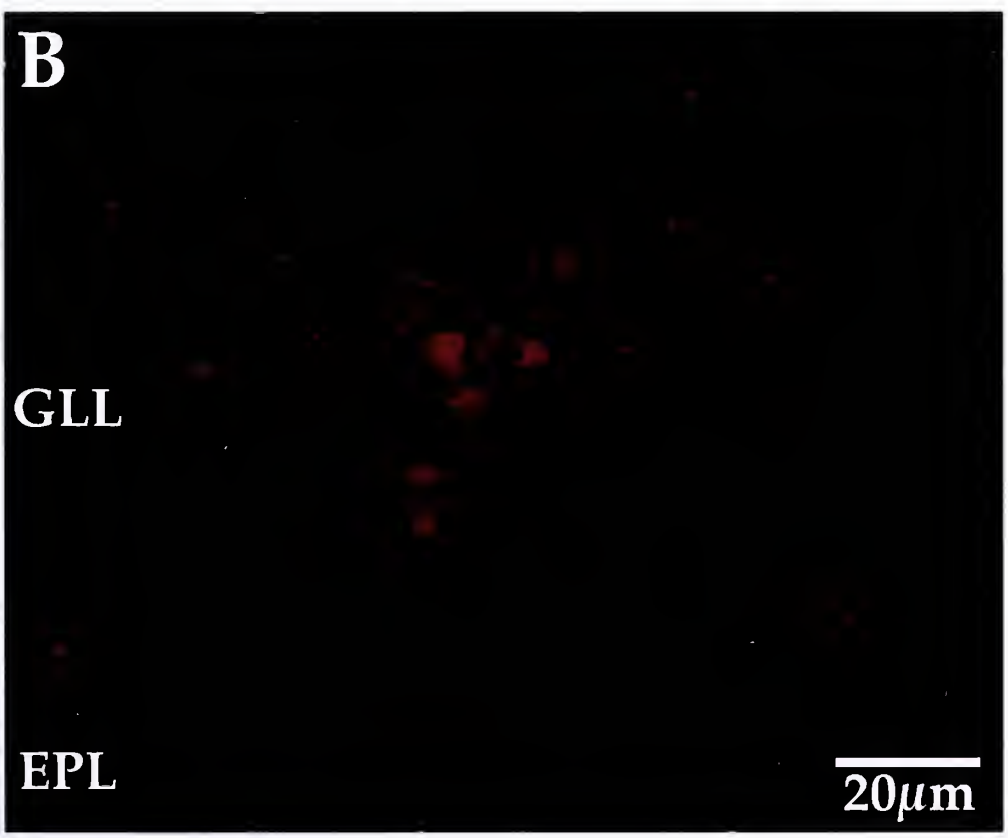
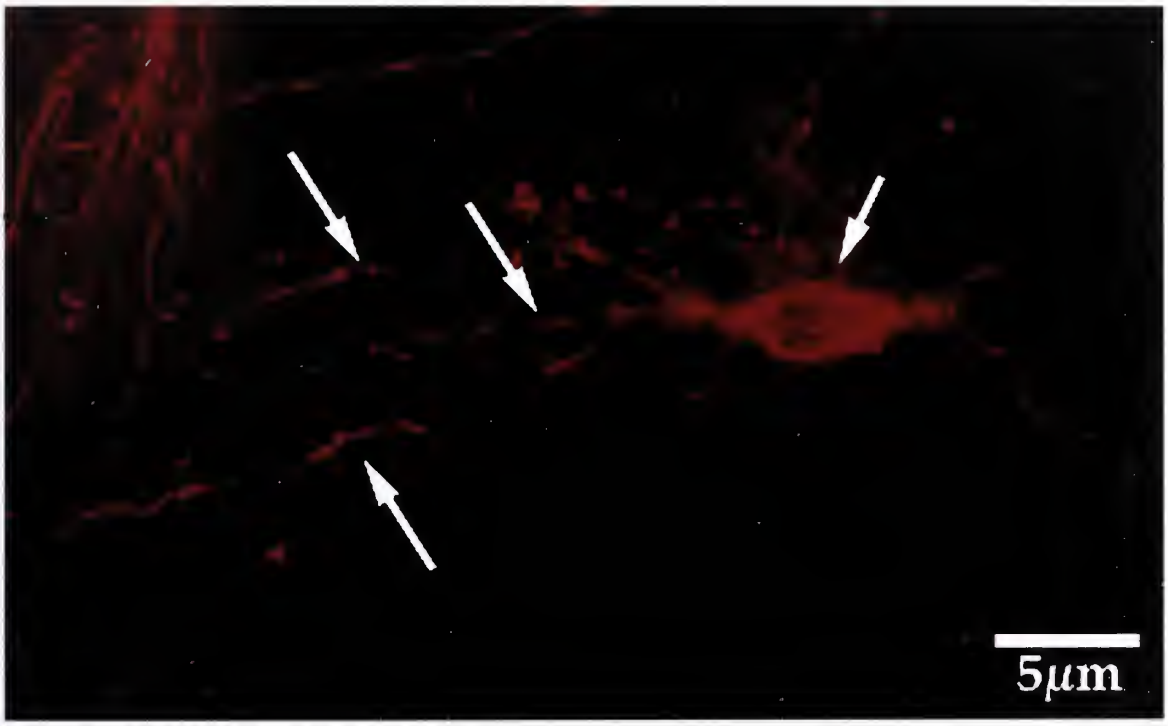

B
GLL
EPL
20μm

Figure 19:

Confocal Microscopy of a DiI Labeled Periglomerular Cell. The clarity with which DiI labels neuronal elements in the olfactory bulb is evident in this figure. The somata (short arrow) and dendritic processes (long arrows) of this cell are easily recognized. Like the terminal arbors of the apical dendrites of mitral and tufted cells, the dendritic arbors of periglomerular cells may also fill the core of the glomerulus. [short arrow=soma; long arrows=dendrites]


5μm

DISCUSSION

DiI Labeling with Confocal Analysis Offers Advantages Over Previous Studies

Prior studies of the distribution of primary olfactory afferents in olfactory bulb glomeruli relied upon the Golgi-impregnation technique. They demonstrated that single olfactory receptor neuron axons occupy only a small fraction of the entire glomerular volume and suggested that primary olfactory afferents do not arborize homogeneously throughout the glomerular neuropil, but instead segregate their arborizations within and target their terminations to discrete sub-compartments (Hálasz and Greer, 1993). The capricious staining characteristics and reliance on light microscopy of the Golgi-impregnation technique, however, limited the analysis of these fibers and impeded further elucidation of their topology. Although the Golgi-impregnation technique stains individual neurons in their entirety, it occurs in a random and unpredictable manner. Thus any possible study is limited to those processes of interest which happen to be labeled. In glomeruli containing the arborizations of multiple stained fibers, the resolving power of light microscopy is insufficient for tracing the course of individual axons, defining their intraglomerular topology, and identifying possible targeting within a mesh of stained processes.

The use of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) labeling and confocal laser microscopy, in the present study, eliminated the limitations imposed by the Golgi-impregnation technique. DiI crystals could be placed directly into the olfactory nerve layer of the olfactory bulb, thereby allowing for specific and extensive labeling of the olfactory

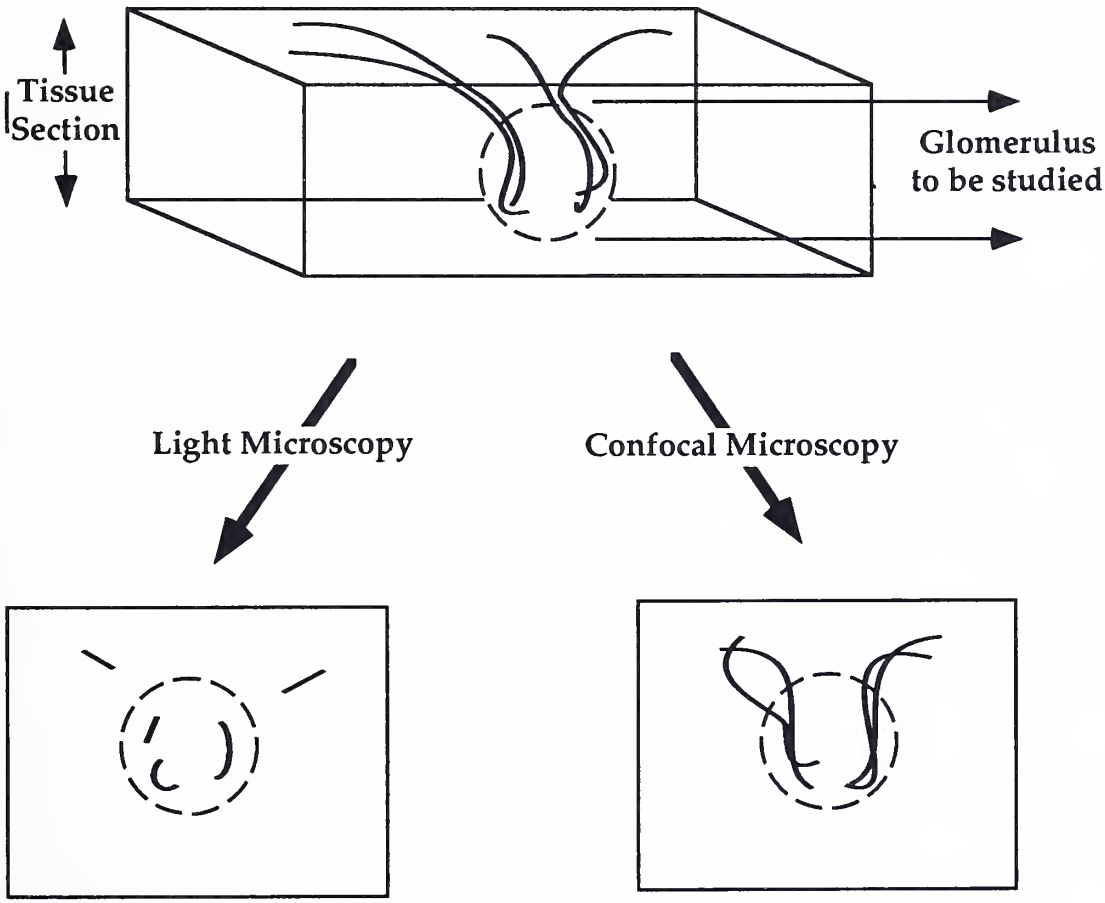
receptor neuron axons under investigation. A more detailed analysis of their topographic distribution was then possible with the superior resolving power of confocal microscopy (Figure 20). In conventional light microscopy, the clarity of a focused image is often obscured by the depth of tissue above and below the plane of focus. Limited to this single focus plane, it cannot display, in one frame, a focused image of the complete course of an axonal process (Left in Figure 20). The Krypton-Argon laser used in confocal microscopy visualizes only those processes in the thin plane at which it is focused, 0.1-Nμm, categorically ignoring all other tissue in the generation of an image. Although the result is not unlike that obtained with light microscopy, only clearer, repeating this process at all levels encompassing a stained fiber of interest produces a unified image of great depth, at high magnification, and all in clear focus (Right in Figure 20). In addition, three-dimensional reconstructions may be produced to facilitate the topological analysis of individual fibers and fascicles. The results obtained with DiI staining and confocal microscopic analysis reveal that this technique is suitable for the study of the topological distribution of primary olfactory afferents as they approach, penetrate, and arborize within individual olfactory bulb glomeruli and complements previous studies using the Golgi-impregnation technique.

Confocal Analysis of DiI Staining

Although individual DiI crystals were intentionally placed into the most superficial layer of the olfactory bulb in order to stain only the primary olfactory afferents, occasional deeper placements and free diffusion from the olfactory nerve layer often yielded images in which the distinctive laminar

Figure 20:

Schematic Diagram Illustrating the Benefits of Confocal Microscopy for Studying the Three-dimensional Anatomy of the Distribution of Olfactory Receptor Neuron Axons in Olfactory Bulb Glomeruli. The resolving power of light microscopy produces a single focused plane within a tissue section. Only the structures in that particular layer are brought into clear focus and able to be visualized (Depicted on Left). Conventional light microscopy cannot display, in one frame, a focused image of the complete course of an axonal process which passes throughout the depth of a tissue section. The Krypton-Argon laser used in confocal microscopy can be focused at extremely thin lamina within a tissue section to construct an image inclusive of only those structures in clear focus and absent of everything else above or below the plane of focus. Although this produces images not unlike those obtained with light microscopy, repeating this process at all levels encompassing a stained fiber of interest and displaying the individual frames simultaneously produces a unified image of great depth, at high magnification, and all in clear focus (Depicted on Right).


Tissue
Section
Glomerulus
to be studied
Light Microscopy
Confocal Microscopy

organization of the olfactory bulb as well as the cyto-architectural features of its individual neuronal elements were demonstrated. The coincidental staining of primary olfactory afferent fibers and the processes of mitral, tufted, periglomerular, and granule cells is evident in Figure 9. Easily traced throughout their length, these neurons follow the precise paths outlined in the earliest anatomical studies of the olfactory bulb (Cajal, 1911). Primary afferent fibers (short arrows) are identified within the olfactory nerve layer (ONL) and penetrating individual glomeruli (asterisks). The apical dendrites (long arrows) of mitral or tufted cells can be seen ascending through the external plexiform layer (EPL) and then ramifying within these same glomeruli. Although the cellular contributions to the glomerular neuropil are thus demonstrated colocalized in single glomeruli, the ability to resolve the topological distribution of primary olfactory afferents is difficult with such heavy labeling. Hence, such glomeruli were not selected for more detailed analysis (See discussion of selection criteria in Materials and Methods). The ability of DiI to label all of the individual cellular elements of the olfactory bulb in a recognizable manner, however, is certainly apparent.

When individual olfactory receptor neuron axons were singly stained in a glomerulus, they could be studied with ease (Figure 10 and 11). Single axons were demonstrated to penetrate individual glomeruli and then undergo arborization patterns of varying tortuousity and complexity, with intraglomerular axonal collaterals ranging from 2-6. Along their course, axonal varicosities (long arrows) and terminal boutons (short arrows), the sites of synaptic interactions, are demonstrated. Restricting their terminal arbors to a limited region within a glomerulus, the axons of olfactory receptor neurons were not observed to arborize throughout the glomerular neuropil. Indeed, only a fraction of the total glomerular volume appears to be occupied

by the terminal arbors of any single olfactory receptor neuron axon. These observations are consistent with Golgi-impregnated samples and confirms the presumption that DiI is labeling the same population of primary afferent fibers analyzed in previous studies. Upon tracing these axons back to their site of origin in the olfactory nerve layer, there was no evidence to suggest branching of primary olfactory afferents prior to entering the confines of a single glomerulus, as observed by other investigators (Allison and Warwick, 1949; Pinching and Powell, 1971a, 1971b).

Individual fascicles were often labeled within the olfactory nerve layer and could be traced into the glomerular layer where they terminate (Figure 12). The relatively enormous distance, from a glomerulus, over which a fascicle could be delineated suggests that olfactory receptor neuron axons destined to arborize in the same glomerulus coalesce early in their course through the olfactory nerve layer. In Figure 12 for example, the glomerulus marked by an asterisk is clearly targeted by a fascicle (arrows) that forms more than 250μm away. Generally following a linear path and without branching, these fascicles appear to target specific glomeruli within the glomerular layer of the olfactory bulb, rather than terminating diffusely across multiple glomeruli (arrows). The development of collaterals by individual fascicles or the commingling of fibers between different fascicles was not demonstrated in the samples studied. The possibility that these fascicles represent the clustering of a related class of olfactory receptor neuron axons is certainly plausible and supported by observations in previous studies. Fascicles of apparently functionally-specific olfactory receptor neuron axons have been identified in the olfactory nerve layer of the olfactory bulb with 2-deoxyglucose (Lancet et al., 1982). In addition, bundles of putative odorant receptor-specific olfactory receptor neuron axons can be identified in the

olfactory nerve layer and traced into the individual glomeruli in which they terminate (Vassar et al., 1994). The observations in DiI labeled specimens together with these previous functional and anatomical investigations thus suggest that functionally related, and perhaps even odorant receptor-specific, axons possess a mechanism to guide them along a path prescribed by their functional or odorant receptor type.

Recent evidence indicates that the odorant receptor protein itself may play a pivotal role in both the formation of these glomerular-specific fascicles and in their maintenance despite the continual turnover of olfactory receptor neurons. Messenger RNA of putative odorant receptors is expressed by olfactory receptor neurons even before they form synaptic interactions (Strotmann et al., 1995) and can be identified within intraglomerular terminals throughout the lifetime of the cell (Ressler et al., 1994; Vassar et al., 1994). Although the presence of translated odorant receptor protein within these terminals remains to be authoritatively demonstrated, investigators have already elucidated a possible cell-surface identification role for these putative odorant receptors (Singer et al., 1995). Correlation mutation analysis has revealed a correspondence between the amino acid structure of the odorant binding pocket in an odorant receptor and the sequence of a certain extracellular loop (E2), rich in integrin-like sequence motifs (Singer et al., in press). Thus the expression of a specific odorant receptor by an olfactory receptor neuron not only establishes its functional responsiveness, it may also serve to guide the developing axon to the appropriate targets.

Within the glomerulus, the terminal arbors of those axons from the same fascicle do not distribute homogeneously throughout the glomerular neuropil, but rather appear to limit their ramifications to a spatially restricted domain within the glomerulus. This is clearly demonstrated in those

glomeruli penetrated by two distinct fascicles of olfactory receptor neuron axons (Figures 13, 14, and 16). The terminal arbors of those axons from a particular fascicle are colocalized within a limited region of the glomerulus, segregated from the terminal arbors of those axons from a different fascicle, thus forming fascicle-specific sub-compartments within the glomerulus (asterisks in Figure 13). Gross overlap and intermingling of axons from different fascicles was not observed in the samples studied. Only along the borders of these sub-compartments, where there appeared to be a slight overlap, was there minimal interdigitation of component fibers from distinct sub-compartments. In three-dimensional reconstructions of these glomeruli (Figures 15 and 16B), this sub-compartmental topology was further defined. The axons of olfactory receptor neurons appear to concentrate their ramifications within the peripheral shell of the glomerulus, while the core of the glomerulus is relatively free of the arbors and terminals of olfactory receptor neuron axons (asterisks in Figure 15). This fascicle-specific sub-compartmentalization within the glomerulus suggests parallel pathways from the neuroepithelium to the olfactory bulb, each forming independent synaptic interactions with unique sets of post-synaptic bulbar elements. The tortuous course followed by some primary olfactory afferents as they ramify within these glomerular sub-compartments suggests highly specific targeting for discrete post-synaptic, second-order processes (Figure 11 and arrowheads in Figures 16 and 17).

Singly labeled principal or periglomerular cell dendritic arbors within a glomerulus provided the opportunity to study the arborization pattern of the second order neurons with which primary olfactory afferents form synaptic contacts in the olfactory bulb glomerulus. While periglomerular cells (Figure 19) send a variable number of dendritic branches into a glomerulus, the

ramification pattern of mitral and tufted cells (Figure 18) appears more stereotypic. After penetrating into the glomerular neuropil, the apical dendrites of principal neurons successively branch in two, producing four terminal limbs from which complex arbors pervade radially. Occupying a central position in the core of the glomerulus, these processes appear to overlap the terminal arbors of olfactory receptor neuron axons mostly where they extend into the glomerular shell.

Confocal analysis of DiI stained olfactory receptor neuron axons, as they approach, penetrate, and arborize within the glomeruli of the olfactory bulb indicates a specificity of targeting by individual afferent fibers and a sub-compartmental organization within the glomerular neuropil, not appreciated in previous studies in the rat. Individual axons, perhaps functionally or putative odorant receptor related, coalesce into glomerular-specific fascicles that target discrete glomeruli. Within the glomerulus, the axonal collaterals of single olfactory receptor neuron axons perform tortuous turns on serpentine paths to presumably discrete post-synaptic elements. Occupying only a limited proportion of the glomerular volume, the region innervated by the terminal arbors of any single olfactory receptor neuron is variable in size and shape. Yet despite this seeming variability at the level of single axons, fascicles of primary afferent fibers clearly establish a precise topological organization within the glomerulus. Axons which share a common fascicular origin arborize within a spatially restricted region of the glomerulus mostly segregated from the fibers of a different fascicle, thus forming fascicle-specific sub-compartments. In addition, the terminal arbors of olfactory receptor neuron axons appear to be concentrated in the peripheral shell of the glomerulus, while the glomerular core is occupied mostly by the apical dendritic processes of principal and periglomerular cells. The olfactory bulb

glomerulus thus possesses a sub-compartmental organization (Figure 21) in which the terminal arbors of a related class of olfactory receptor neuron axons intermingle with a specific sub-set of dendritic processes within spatially restricted regions of the glomerular neuropil. Since synaptic interactions are possible only between overlapping fibers, the olfactory bulb glomerulus appears to contain at least two, and perhaps multiple, sites of independent odorant processing.

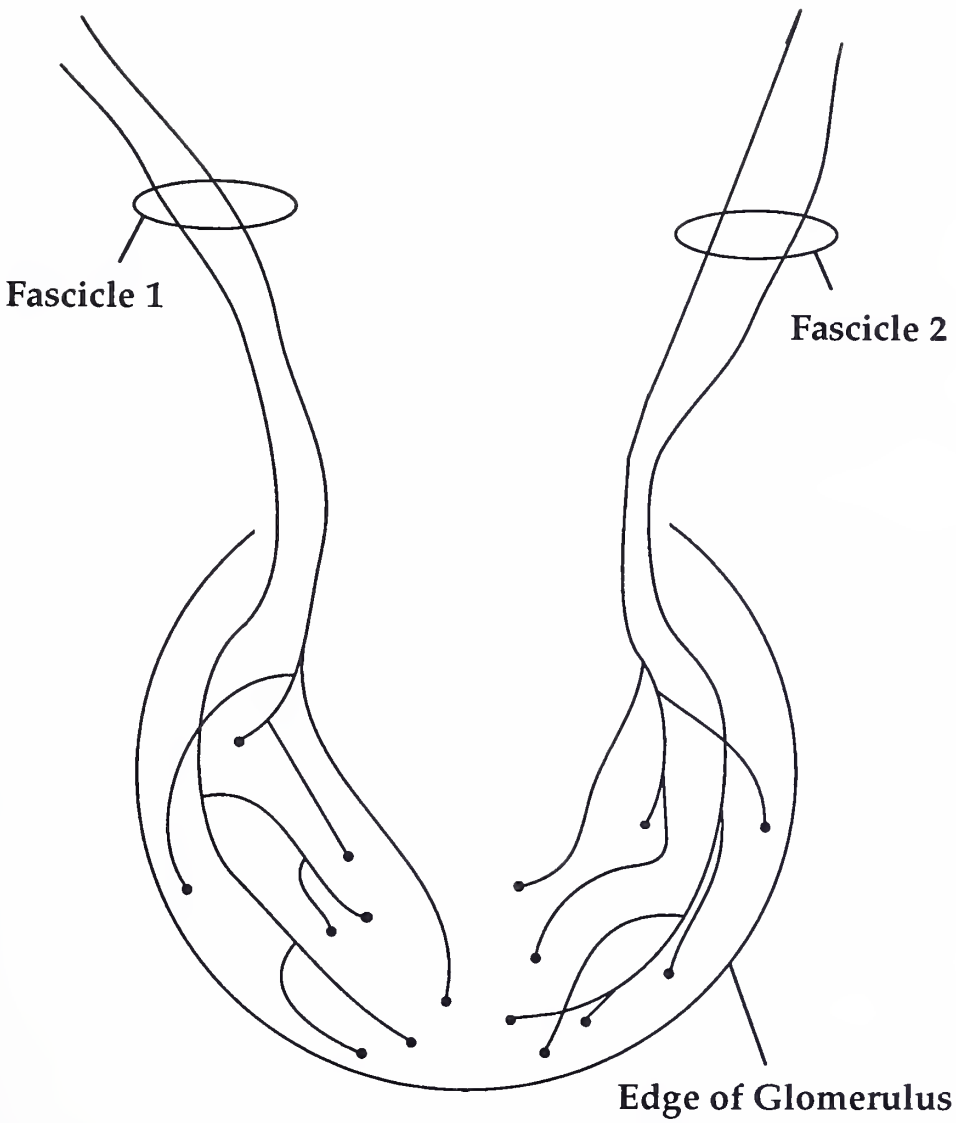
Corroborating Evidence for Sub-compartmentalization from Invertebrates

The olfactory system of many invertebrate species encompasses the same general framework observed in vertebrates. Initial odorant detection involves the interaction of stimuli with the terminal dendrites of olfactory receptor neurons in highly specialized sensory organs, the antenulles. Via the antennular nerve, the axons of olfactory receptor neurons project to the central nervous system and terminate in discrete, columnar-shaped regions of neuropil in the olfactory lobe. Within these structures, the primary afferent fibers form synaptic interactions with the terminal dendritic arbors of intralobar interneurons and second-order projection neurons. The compartmental segregation of the first synapse in olfactory processing in olfactory glomeruli thus appears to represent a key feature of the olfactory pathway and is retained across species.

Investigations directed at tracing the projections of olfactory receptor neuron axons into the olfactory lobe of the spiny lobster, *Panulirus argus*, have demonstrated a sub-compartmental organization of their terminal arbors in olfactory glomeruli (Schmidt and Ache, 1992). Although single

Figure 21:

Schematic Diagram Illustrating the Topological Distribution of Olfactory Receptor Neuron Axons in Olfactory Bulb Glomeruli. Olfactory receptor neuron axons from the same fascicle appear to arborize within spatially segregated regions of the glomerulus, separate from the terminal arbors of those axons from a different fascicle. In addition, the terminal arbors of olfactory receptor neurons appear more heavily concentrated in the peripheral shell of the glomerulus with less dense innervation of its central core. This suggests a level of parallel processing within the glomerulus in which discrete pathways from the neuroepithelium could undergo individualized processing within the same glomerulus.


Fascicle 1
Fascicle 2
Edge of Glomerulus

axons ramify throughout the confines of a glomerulus, there is an apparent concentration of primary afferent fibers in the cap/subcap and the innermost portion of the base in these columnar glomeruli. In comparison, the region of the base between these two zones appears conspicuously deficient of the axons of olfactory receptor neurons. Terminal boutons and en passant varicosities, the sites of synaptic interactions, were similarly localized to the cap/subcap and innermost portion of the base in a seemingly preferential manner. Primary afferent input to the olfactory glomerulus is thus segregated in two distinct zones which may be engaged in differential processing of odorant information with unique sets of post-synaptic elements. Indeed particular classes of intralobar interneurons and projection neurons have been demonstrated to distribute their terminal dendritic processes within either the cap/subcap or innermost portion of the base, supporting the regionalization of distributive processing within a single glomerulus (Schmidt et al., 1991; Schmidt and Ache, 1992).

Recognizing that this sub-compartmental organization of the olfactory glomerulus was a novel discovery at the time of publication of the aforementioned study, the conclusions reached by its authors are not surprising:

> Superficially, at least, regionalization of function in arthropod glomeruli would appear to differ from the vertebrate plan, where afferent branches and processes of secondary neurons are generally distributed evenly throughout olfactory glomeruli (Schmidt and Ache, 1992).

On the contrary, the axons of olfactory receptor neurons in vertebrates segregate their terminal arbors to discrete spatial domains, defined by their fascicular origin, within the olfactory bulb glomerulus. Two sub-compartments were commonly identified in the olfactory bulb glomeruli of

the rat, synonymous with the dichotomous distribution of primary afferents in the olfactory glomeruli of the spiny lobster. The conservation of this organizational scheme by evolution, across species, presumes its key role in olfactory processing and necessitates a reevaluation of the functional organization of the olfactory glomerulus.

Parallel Pathways of Primary Afferent Projections to the Olfactory Bulb

Parallel processing of sensory information is a common theme across sensory systems. Vision relies upon two distinct receptor types, rods and cones, for the differential processing of black-and-white and color stimuli. Modified by synaptic interactions with unique sub-sets of higher-order neurons, the information encoded by each receptor type remains segregated in distinct but parallel projections to visual cortex. The somatosensory system uses multiple receptor types, each tuned to a particular physical stimulus, and each projecting to a functionally specific region of the homunculus in somatosensory cortex. Although parallel pathways have not been clearly delineated in the olfactory system, corollaries have been proposed from anatomical and physiological observations.

The sub-laminar distribution of dendritic processes of sub-populations of mitral, tufted, and granule cells in the external plexiform layer appears to represent the segregated processing of two parallel pathways. While the apical dendrites of superficial granule cells and the secondary dendrites of tufted cells are colocalized within a superficial stratum of the external plexiform layer, deeper laminae contain the overlapping processes of deep granule cells and mitral cells. Highly specific dendrodendritic interactions are

identified among the overlapping fibers in these two sub-lamina of the external plexiform layer. Thus while superficial granule cells appear to control the activity of tufted cells, mitral cells are influenced by the inhibitory output of deep granule cells. Although the axons of mitral and tufted cells together carry the efferent activity from the olfactory bulb to cortical structures involved in odorant processing, the specificity with which each population forms synaptic interactions with a particular sub-population of intrabulbar interneurons in segregated domains within the olfactory bulb suggests that these two populations represent parallel pathways involved in differential odorant processing (Shepherd and Greer, 1990). Indeed synonymous with parallel pathways in other sensory systems, the cortical projections of mitral and tufted cells to piriform cortex appear to obey a strict topography (Scott, 1981; Prince, 1987).

The degree to which these parallel pathways segregate within the olfactory bulb glomerulus and the nature of their primary afferent input remains to be elucidated. Although the notion that the apical dendrites of mitral and tufted cells receive identical afferent input and then perform differential processing via their interactions with unique sub-sets of granule cells is conceivable, the sub-compartmental organization in which primary afferents are distributed within the glomerulus could suggest differential input to each population of principal neuron. This is supported by the common observation, among the glomeruli studied, of a single glomerulus penetrated by two distinct fascicles and thus containing two segregated sub-compartments. While the primary afferent input via one fascicle may form synapses exclusively with mitral cells, the terminal arbors of those axons from the other fascicle would interact wholly with tufted cells. As in other sensory systems, these parallel pathways from the neuroepithelium to the

olfactory bulb may represent the simultaneous processing of multiple independent qualities of a particular stimulus. Highlighting a functional specificity for these afferent fascicles is clearly necessary to further support this proposal. In addition, future investigation will need to address the possibility that the terminal arbors of apical dendrites of mitral and tufted cells distribute within an intraglomerular topology in a complementary manner to the sub-compartmental organization of the axons of olfactory receptor neurons.

Multiple Odorant Specificity Within Individual Glomeruli

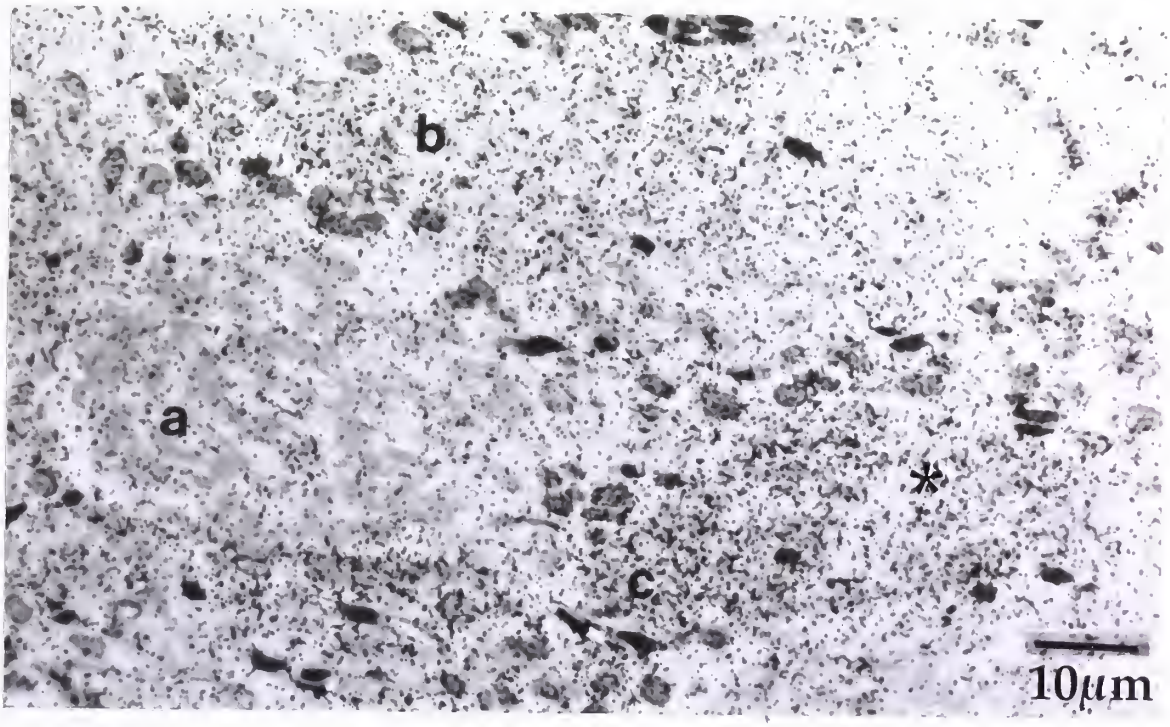
The discovery of a multigene family, belonging to the superfamily of seven transmembrane G-protein linked receptors, whose expression is limited to the olfactory receptor neurons of the neuroepithelium has significantly contributed to the elucidation of the discriminatory code employed by the olfactory system (Buck and Axel, 1991; Ressler et al., 1993). Aware of the G-protein dependent second messenger cascade involved in odorant transduction, many authors have postulated that the transcripts of these genes may represent the odorant receptor proteins expressed on the cilia of olfactory receptor neurons, which bind odorant epitopes and trigger primary afferent signals. In addition, each olfactory receptor neuron is believed to express about one of the approximately 100 members of this putative odorant receptor gene family (Ngai et al., 1992; Ressler et al., 1993) and thus possess a fine-tuned responsiveness to a limited number of related odorant epitopes. Although the distribution of odorant-receptor specific olfactory receptor neurons is apparently random within a zone of the neuroepithelium (Ressler et al., 1993), their axons clearly converge onto

specific, individual glomeruli in the olfactory bulb (Vassar et al., 1994). Hence, the activity of any single olfactory bulb glomerulus appears to be odorant epitope-specific. Since each odorant is a conglomeration of a unique set of epitopes, the discriminatory code employed by the olfactory system could involve the singularly unique pattern of activity across the glomerular layer elicited by a particular stimulus. The olfactory bulb glomerulus, as a whole, is thus considered the fundamental unit of this code.

The anatomical and physiological studies which support this model, however, have not addressed the possibility of multiple odorant specificity within a single glomerulus. Convergence of odorant receptor-specific olfactory receptor neuron axons upon individual glomeruli has been demonstrated in two complementary studies: (1) in-situ hybridization with probes against the primary transcripts of putative odorant receptor genes (Vassar et al., 1994) and (2) by tau-lac-Z mice. Although both studies identified apparently uniformly labeled glomeruli, the level of resolution is perhaps insufficient to conclude consistent labeling of all primary afferent processes. In addition, the use of single probes obviated any possible observation of multiple odorant receptor-specific regions within a particular glomerulus. Functional studies with 2-deoxyglucose (Lancet et al., 1982) which have been previously interpreted to indicate homogeneous responses throughout the glomerular neuropil, actually demonstrate sub-compartments of activity within individual glomeruli. In Figure 22, the presentation of a particular odorant elicits increased levels of activity in only the left half of glomerulus c. The activity in the right half (asterisk) appears indistinguishable from the background. This regional activity suggests differential primary afferent input to two distinct sub-compartments of the glomerulus.

Figure 22:

Odorant-induced Glomerular Activity Reflected by 2-Deoxyglucose Uptake. Although 2-deoxyglucose competes with glucose and is therefore avidly absorbed by active cells, its failure to undergo complete hydrolysis results in intracellular accumulations which can be localized with autoradiography. The silver graining in this figure thus represents active cells, and its density can be interpreted as a reflection of regional activity. The three glomeruli in this figure each depict a different level of activity in response to odorant presentation: (a) activity less than background, (b) activity comparable to background, and (c) activity greater than background. Closer inspection, however, reveals that the activity in glomerulus c is not uniformly greater than background, but instead appears to contain an active sub-compartment on the left (c) and a region on the right (asterisk) where the activity is comparable to background. One explanation for this regional activity is differential odorant input to each sub-compartment of the glomerulus. (Adapted from Lancet et al., 1982)


b
a
*
c
10μm

In the glomeruli analyzed in the present study, two sub-compartments were indeed the most common observation.

The sub-compartmental organization of the olfactory bulb glomerulus could thus represent the segregation of processing of distinct odorant information within a single glomerulus. Two fascicles penetrating a single glomerulus could each transmit a singularly specific odorant response from the neuroepithelium to a unique set of post-synaptic elements overlapping its terminal arbors in its specific glomerular sub-compartment. The result is a two-fold increase in the number of odorant-specific units contributing to the generation of odorant-induced patterns of activity across the glomerular layer, and therefore a more refined odortopic map. Although these functional characteristics have yet to be attributed to glomerular sub-compartments, preliminary future studies must investigate the odorant-receptor specificity of the primary afferents to a single glomerulus with greater specificity and higher resolution than previously employed. Using double labeling, the odorant-receptor specificity of each fascicle penetrating a single glomerulus can be assessed. Within the glomerulus, electron microscopy will be an invaluable technique to identify the labeling of primary afferents by a single or multiple in-situ probes. The continued development of highly specific experimental olfactory stimuli could fuel physiological studies and further an understanding of possible differential activity within a glomerulus.

CONCLUSIONS

The single most important observation to emerge from the confocal analysis of DiI stained olfactory receptor neuron axons is the sub-compartmental organization of the olfactory bulb glomerulus. Single olfactory receptor neuron axons, destined to arborize in the same glomerulus, coalesce into glomerular-specific fascicles during their passage through the olfactory nerve layer, target and penetrate specific, individual glomeruli, and arborize within spatially restricted regions of the glomerular neuropil, topologically defined by their fascicular origin. In addition, olfactory receptor neuron axons appear to concentrate their terminal arbors in the peripheral shell of the glomerulus with few fibers penetrating into the glomerular core. While the functional significance of these glomerular sub-compartments remains to be elucidated, their preservation across species suggests an essential role for sub-compartmental processing in odorant detection, discrimination, and recognition. Glomerular sub-compartments may represent the segregation of parallel afferent pathways from the neuroepithelium to the olfactory bulb, or alternatively, glomerular sub-compartments could be involved in the individualized processing of differential odorant information within a single glomerulus.

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